UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

No. 2013-1011, -1029, -1376 PROMEGA CORPORATION,

Plaintiff-Cross Appellant,

and

MAX-PLANCK-GESELLSCHAFT ZUR FORDERUNG DER WISSENSCHAFTEN E.V.,

Plaintiff,

V.

LIFE TECHNOLOGIES CORPORATION, INVITROGEN IP HOLDINGS, INC., and APPLIED BIOSYSTEMS, LLC,

Defendants-Appellants.

On Appeal from the United States district court for the Western District of Wisconsin, Case No. 3:10-cv-00281-bbc, Hon. Barbara B. Crabb

OPENING BRIEF OF DEFENDANTS-APPELLANTS NONCONFIDENTIAL

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CERTIFICATE OF INTEREST

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Biosystems, LLC, and Invitrogen IP Holdings, Inc. certifies as follows:

1. The full name of every party or amicus represented by us is:

Life Technologies Corporation Applied Biosystems, LLC Invitrogen IP Holdings, Inc.

2. The name of the real parties in interest represented by us are:

Life Technologies Corporation Applied Biosystems, LLC Invitrogen IP Holdings, Inc.

3. All parent corporations and any public companies that own 10 percent or more of the stock of the party represented by us are:

Life Technologies Corporation does not have a parent corporation, nor does any public company own more than 10 percent of its stock. Applied Biosystems, LLC and Invitrogen IP Holdings, Inc., are wholly owned subsidiaries of Life Technologies Corporation.

4. The names of all law firms and the partners or associates that appeared for the parties now represented by us in the trial court or are expected to appear in this court are:

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CONFIDENTIAL MATERIAL OMITTED

The material omitted on pages 20 and 21 contains testimony designated as confidential by Promega, describing Promega's research and development process; the material omitted on pages 26 and 51 contains the terms of a confidential license agreement.

TABLE OF AUTHORITIES

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TABLE OF ABBREVIATIONS AND CONVENTIONS

Ax(:y) page x of the Joint Appendix (at line y)

Life Life Technologies Corp., Invitrogen IP

Holdings, and Applied Biosystems, LLC

(collectively)

IP Holdings Invitrogen IP Holdings

AB Applied Biosystems, LLC

Promega Corporation

'984 patent/ U.S. Patent No. RE 37,984

Tautz patent

'660 patent U.S. Patent No. 5,843,660

'598 patent U.S. Patent No. 6,221,598

'771 patent U.S. Patent No. 7,008,771

'235 patent U.S. Patent No. 6,479,235

Promega patents '660 patent, '598 patent, '771 patent, and

'235 patent (collectively)

2006 Cross License Cross License Agreement dated August

29, 2006 between Promega Corporation and Applera Corporation, through its

Applied Biosystems Group

STR Short tandem repeat

PCR Polymerase chain reaction

STATEMENT OF RELATED CASES

In a prior appeal, this Court affirmed the district court's grant of Defendant Invitrogen IP Holdings, Inc.'s ("IP Holdings") motion to compel arbitration for some of the claims in this case. *See Promega Corp. v. Life Technologies Corp.*, 674 F.3d 1352 (Fed. Cir. 2012) ("*Promega I*"). The outcome of that arbitration could affect or terminate Promega's rights to one of the five patents at issue in this appeal, U.S. RE 37,984 (the "Tautz patent"). *See id.* at 1358. The case *Promega Corp. v. Applied Biosystems LLC*, No. 1:13-cv-02333 (N.D. Ill. Mar. 22, 2013), for which a notice of appeal to this Court was filed on June 26, 2013, includes a counterclaim that Promega Corporation ("Promega") breached a 2006 Cross License Agreement that is also at issue in this appeal. Counsel knows of no other case pending in this Court or any other court that may directly affect, or be directly affected by, the Court's decision in this appeal.

STATEMENT OF JURISDICTION

The district court had original jurisdiction pursuant to 28 U.S.C. §§ 1331 and 1338(a). Life Technologies Corporation, Invitrogen IP Holdings, Inc., and Applied Biosystems, LLC (collectively, "Life") timely appealed from the district court's final judgment on September 27, 2012 that disposed of all parties' claims. A2355 [Notice of Appeal]. The appeal was consolidated with Appeal No. 2013-1029 filed by Promega. Dkt. No. 6. That consolidated appeal was deactivated when Promega

moved to amend the underlying judgment, for a new trial and for relief from the judgment. Dkt. No. 16.¹ Following the denial of those motions, the appeal was reactivated and consolidated with another appeal filed by Promega (Case No. 2013-1376). Dkt. Nos. 20, 24.

Life appeals from the district court's September 18, 2012 Amended Judgment (A70) dismissing Life's counterclaims for a declaratory judgment that U.S. Patent Nos. 5,843,660, 6,221,598, 6,479,235, and 7,008,771 are invalid and licensed. A2355 [Notice of Appeal]. Jurisdiction over Life's invalidity counterclaim is proper under 28 U.S.C. § 1295(a) notwithstanding that the judgment rejected Promega's infringement claims. *See Fort James Corp. v. Solo Cup Co.*, 412 F. 3d 1340, 1348 (Fed. Cir. 2005) (finding appeal of invalidity issue proper despite finding of non-infringement, because "a counterclaim questioning the validity or enforceability of a patent raises issues beyond the initial claim for infringement that are not disposed of by a decision of non-infringement.").

Applied Biosystems, LLC ("AB") also appeals from the district court's Amended Judgment (A70-71) insofar as it rejected AB's declaratory judgment counterclaim that it is validly licensed to practice the Promega patents. A2355 [Notice of Appeal]. Jurisdiction over AB's license counterclaim is proper under 28

¹ Citations to the docket refer to entries in the lead appeal, Case No. 13-1011.

U.S.C. § 1295(a) because the counterclaim seeks relief broader than the denial of Promega's claims of patent infringement in this case. AB's license counterclaim seeks a declaration that AB has a license to practice the asserted patents as a whole, for current and future products. *See* A567 [Defs.' Answer] ¶¶ 19-23; *Board of Trustees of Leland Stanford Junior University v. Roche Molecular Systems, Inc.*, 583 F.3d 832, 839 (Fed. Cir. 2009) (finding appeal of license arguments proper despite summary judgment of invalidity, because "Roche's ownership and license arguments would establish Roche's rights to the patents as a whole, not only to specific claims. . . . Roche's arguments would expand its rights under the judgment and, thus, are properly the subject of a cross-appeal."). Correcting the scope of the license would expand AB's rights and reduce Promega's rights. *See* A567 [Def's Answer] ¶¶ 19-23.²

PRELIMINARY STATEMENT

Life and Promega are vigorous competitors in the market for DNA testing kits used to identify criminals and parents, among other applications. The dispute over Promega's patents began over a decade ago. In this appeal, Life establishes

²The license issue is, in any event, properly raised as part of this appellate proceeding because it is also an alternative basis to support the district court's judgment in favor of AB and should be considered as such.

that the Promega patents are invalid and, additionally, that Life is properly licensed in certain disputed fields of use.

Before trial, the district court rejected Life's invalidity counterclaim on summary judgment and also construed the license narrowly against Life. Thus, infringement was tried without the invalidity of the patents or the proper scope of the license being presented to the jury. The jury returned a verdict in favor of Promega and awarded more than \$50,000,000 in damages. Because the trial record failed to support the verdict, the district court granted JMOL and entered judgment in favor of Life on infringement.

Although Life prevailed below on infringement, it appeals the rejection of its invalidity and license claims because those issues have an on-going impact on the parties' commercial relationship. Moreover, insofar as the patents are deemed invalid or licensed, various issues Promega may present in its cross-appeal become moot.

The Promega patents, which have been construed to cover a staggering range of subject matter, are invalid. On the one hand, much of the range of subject matter claimed by the patents has not been enabled. On the other hand, the narrowest embodiments within the broad range of the claims reflect only minor differences achievable through rote application of known methods. Thus the claims are also invalid as obvious. Moreover, Life is licensed not only to supply

labs for courtroom forensic tests, but also for the education, training and research necessary to serve the forensics market.

STATEMENT OF THE ISSUES

- 1. Did the district court err in rejecting on summary judgment Life's proof that the Promega patents are not enabled?
- 2. Did the district court err in rejecting Life's showing that the Promega patents are obvious?
- 3. Did the district court err in ruling that Defendants' are not licensed to practice the Promega Patents in the areas of forensic research, education, and training?

STATEMENT OF THE CASE

I. PRIOR CASE

This dispute has a long history. In 2001 Promega sued Applera Corporation ("Applera"), the predecessor company of defendant AB, for infringement of two of the patents asserted here. *See* A2404-2411 [Dkt. No. 2 in Case No. 3:01-cv-0244-bbc (W.D. Wis. April 24, 2001)]. After Applera filed summary judgment motions for invalidity for non-enablement and obviousness, the case settled. *See id.* at A2412. That settlement was memorialized in part in a 2006 patent license between the parties that gives rise to the licensing issue in this appeal.

II. PRETRIAL PROCEEDINGS

A. PROMEGA'S COMPLAINT

Promega filed this suit against Life in retaliation for Life's demand that Promega pay royalties owed under the 1996 patent license agreement or submit to arbitration. *See Promega I*, 674 F.3d at 1354. Promega included declaratory judgment claims to attempt to avoid arbitration under the 1996 patent license agreement. A438-443 [Complaint] ¶¶ 139-173. Promega also alleged that five patents related to genetic testing were infringed, including the two patents involved in the 2001 case: U.S. Patent No. 5,843,660 (the "660 Patent"), U.S. Patent No. 6,221,598 (the "598 Patent"), 6,479,235 (the "235 Patent"), 7,008, 771 (the "771 Patent") (collectively, the "Promega patents") and U.S. Patent No. Re 37,984 (the "984 Patent," or the "Tautz patent"). A409-410 [Complaint] ¶ 1.3

Promega alleged that the following Life products infringe all five patents-in-suit: AmpFlSTRTM Profiler Plus, AmpFlSTRTM COFiler, AmpFlSTRTM Profiler, AmpFlSTRTM Identifiler, AmpFlSTRTM Yfiler. A417-429 [Complaint] ¶¶ 27, 36, 45, 54, 65. Each product is a kit for genetic testing assembled in Warrington, United Kingdom, and sold worldwide. A2265 [2/13/2012 Trial Tr.] at 40:24-41:7.

³ The Tautz patent is licensed to Promega. *Promega I*, 674 F.3d at 1354.

B. CLAIM CONSTRUCTION AND SUMMARY JUDGMENT

On May 24, 2011, the district court construed several terms in the Promega patents, but reserved construction of the term "a set . . . of loci." A657-658 [Order].

The parties subsequently filed cross-motions for summary judgment. A659 [Promega Mot. for SJ]; A838 [Defs' Mot. for Partial SJ]. The district court granted Life's non-infringement summary judgment motion for claims 25 and 27-31 of the '660 patent. A31-32 [Order]. The district court granted Promega's infringement summary judgment motion for the remaining asserted apparatus claims of the patents (claims 10, 23-24, 27, 33 of the '598 patent, claims 18-19 and 21-23 of the '235 patent, claim 5 of the '771 patent, and claim 42 of the Tautz patent). A32; *see also* A1667 [Order]. It also granted summary judgment that the Promega patents were not invalid on grounds of anticipation, obviousness, or lack of enablement (and denied Life's corresponding summary judgment motion that Promega's patents were invalid as not enabled or obvious). *Id.* The district court denied the summary judgment motions in all other respects.

In its summary judgment ruling, the Court construed the claim term "a set ... of loci." The parties disputed whether the claimed set of loci is "open-ended" (claiming the specific set of loci listed in the claims plus any number beyond those expressly listed) or "closed" (claiming only the specific set of loci specified in the

claims and no more). A8-21 [Order]. The district court construed "a set . . . of loci" to be "open-ended" for each of asserted claims at issue in this appeal. *Id.* Thus, according to the district court, infringement occurs when the set of loci in a product includes each of the loci identified in a patent claim even if the set includes any number of additional loci.

At the same time, the district court ruled that the Promega patents need not enable use of any sets with additional loci, even if they are encompassed by the claims. A27. The district court reached this conclusion by deeming additional loci to be unrecited claim elements, which the court referred to as "unrecited loci." A27-28. In other words, the district court found that the claimed invention included "unrecited loci" for purposes of infringement, but excluded the same "unrecited loci" for purposes of enablement.

In its order, the district court also granted Promega's summary judgment motion that the Promega patents are not obvious (and denied Life's corresponding motion seeking summary judgment of obviousness). A32. With little analysis, the district court concluded that there was no triable issue of fact on the obviousness issue. *Id*.

C. THE ORAL RULING REGARDING THE 2006 CROSS LICENSE

Following summary judgment and shortly before trial, each side submitted a trial brief regarding whether certain uses for Life's accused products were licensed

under the 2006 Cross License Agreement between Promega and Applera Corporation.⁴ A1576 [Defs.' Supp. Brief]; A1647 [Promega's Supp. Brief]. The key issue was whether the term "Forensics and Human Identity Applications" in the 2006 Cross License covered forensic research, education, and training, or was limited to forensic work done by police or forensic labs only on "live" forensic investigations. The district court issued an oral ruling on February 6, 2012, stating that "Forensics and Human Identity Applications" "is not covered by the [2006] license unless it's done right there in the police department." A1792 [2/6/2012 Trial Tr.] at 19:1-4. Life was thus precluded from arguing that the disputed forensic research, education and training uses for its accused products were licensed. For instance, Life was precluded from arguing at trial that the uses by at least 15 forensic DNA training institutions accredited by the American Academy of Forensic Sciences were within the scope of the 2006 cross license.

After the district court's pretrial rulings, the issues remaining for trial were (1) the scope of the infringing acts, (2) damages; and (3) willful infringement.

III. TRIAL

Trial was held in February 2012. Because Life assembles its accused kits in the United Kingdom and sells them worldwide, Promega had to prove infringing

⁴ Applied Biosystems through a series of corporate transactions is the successor-ininterest to Applera and is a defendants-appellant here.

acts and damages for particular sales either under 35 U.S.C. § 271(f)(1), for "inducing" foreign assembly of infringing kits with components supplied from the U.S., or under 35 U.S.C. § 271(a), because the products were imported or sold in the U.S.

Despite the particular statutory requirements of Section 271, Promega chose at trial to "rel[y] on the assumption that *all* of the accused products defendants sold during the relevant time frame . . . were made in the United States, imported into the United States or made with a substantial portion of components from the United States, as required by § 271(a) and (f)(1)." A2334 [Order] (emphasis in original). In the words of the district court, Promega "took an 'all or nothing' approach at trial," by seeking to capture damages for all worldwide sales of Life's accused products, based only on United States patents, and without meeting the requirements of Section 271. A2359 [Order]. It did so despite Life's Rule 50(a) motion that provided "fair warning that plaintiff should come forward with [statutorily sufficient evidence for each sale] before submitting its case to the jury." The jury accepted Promega's "all or nothing" position, and A2342 [Order]. returned a verdict of \$52,009,941. A203 [Verdict].

IV. POST-TRIAL PROCEEDINGS

Both parties filed post-trial motions. Life filed a renewed motion for judgment as a matter of law challenging Promega's failure of proof under § 271(a)

and (f)(1), *i.e.*, its attempt to capture damages for all worldwide sales of Life's accused products based only on United States patents without having met the statutory requirements. A2296 [Defs.' Rule 50(b) Mot.].⁵ The district court granted judgment as a matter of law in Life's favor, concluding that Promega "failed to submit admissible evidence at trial showing that all the sales at issue satisfied [the] requirements" of § 271(a) or (f)(1). A2334 [Order]. The district court also concluded that Promega had waived any argument for a new trial on damages by not timely seeking a new trial. A2353 [Order].

In response to the JMOL order, Promega filed another wave of post-trial motions. *See* A2360 [Order]. For the first time, Promega sought a new trial to permit it to abandon its "all or nothing" approach, and seek a lesser damages award from the jury. A2365 [Order]. The district court ruled that Promega's new trial motion was untimely and forfeited. A2365-2366 ("If plaintiff believed that the evidence at trial could support a lesser damages award, it could have and should have raised that issue in response to defendants' Rule 50 motion."). Promega also sought extraordinary relief from judgment under Rule 60 alleging that there was "newly discovery" evidence. A2368. The district court denied the Rule 60

⁵ Promega also filed motions seeking enhanced damages, attorney fees, costs, and a permanent injunction. A2334, A2338-2353 [Order].

motion as untimely, irrelevant, and unpersuasive. A2368-2369 (finding Promega did not file its Rule 60 motion until several months after the applicable deadline, and that in any event Promega failed to show that the evidence would "have made any difference").

In sum, the district court entered judgment: (1) granting Life's motion for partial summary judgment of noninfringement of the '660 patent; (2) granting Promega's motion that the Promega patents are not invalid; (3) dismissing Life's counterclaims; and (4) granting Life's motion for judgment as a matter of law regarding 35 U.S.C. § 271(a) and (f)(1).

STATEMENT OF THE FACTS

I. THE PARTIES

A. LIFE TECHNOLOGIES CORPORATION

Life Technologies is a global life sciences company of over 10,000 employees in 180 countries headquartered in Carlsbad, California. A770 [Defs.' Answer to Second Amended Complaint] ¶ 1. It offers products related to cell biology, genetic analysis, molecular biology, agricultural biology, food safety, human identification, genomic medicine, and other areas.

B. APPLIED BIOSYSTEMS, LLC

Applied Biosystems is a wholly owned subsidiary of Life Technologies Corp. A770 [Defs.' Answer to Second Amended Complaint] ¶ 2. AB focuses on integrated systems for genetic analysis, including products for DNA quantification,

cloning, and delivery into cells, fluorescent dyes, acoustic focusing, molecular diagnostic assays, chromatography, and cancer markers.

C. INVITROGEN IP HOLDINGS, INC.

Invitrogen IP Holdings is a wholly owned subsidiary of Life Technologies. A739 [Defs' Answer to Second Amended Complaint] ¶ 21. IP Holdings holds the rights originally held by Research Genetics, Inc. under a 1996 patent license agreement between Research Genetics, Inc. and Promega. *Promega I*, 674 F.3d at 1354.

D. PROMEGA CORPORATION

Promega is a Wisconsin corporation that offers products in the fields of genomics, protein analysis and expression, cellular analysis, drug discovery and genetic identity. Promega considers Life its "main competitor." A1866 [2/7/2012 Trial Tr.] at 61:13-14; A2323 [Promega Brief re: Injunction].

E. MAX-PLANCK-GESELLSCHAFT ZUR FORDERUNG DER WISSENSCHAFTEN E.V.

Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. is a non-profit German research organization that is the owner of the Tautz patent, which Promega asserts in this case in Promega's capacity as exclusive licensee in limited fields. A739 [Defs.' Answer to Second Amended Complaint] ¶ 18. Max-Planck is an involuntary plaintiff. A596 [Second Amended Complaint] ¶ 18.

II. THE TECHNOLOGY

A. SCIENTIFIC BACKGROUND

DNA is a double-stranded molecule consisting essentially of two complementary strands of nucleotides. A955-956 [Struhl Report] ¶ 3; A978 [Booker Report] ¶ 3. The four nucleotides which are found in DNA are adenine (A), thymine (T), guanine (G), and cytosine (C). A978. Certain regions of DNA contain repeats of a particular nucleotide sequence. For example, the DNA sequence ATT (adenine-thymine-thymine) may be repeated ten times in a row in a particular location. Such repeating regions are called "short tandem repeats," or STRs, and the region in which they occur is called an STR locus. A978. STR loci occur frequently in the human genome—it has been estimated that there are over two million. A226 ['660 patent] at 1:37-39.

The number of repeats of a given sequence at a particular STR locus varies from individual to individual. A956 [Struhl Report] ¶ 4; A979 [Booker Report] ¶ 4. For example, one individual's DNA may have eleven ATT repeats at a given STR locus, while another individual may have fourteen at the same locus. A979. These variations are referred to as "alleles" of the particular locus. A979.

Individuals have unique sets of alleles. STR profiling involves determining the unique set of alleles at multiple STR loci in an individual's DNA to create a DNA "fingerprint," or profile, of an individual. A956 [Struhl Report] ¶ 5; A979

[Booker Report] ¶ 5. STR profiles can be used to match an individual's DNA with a sample of DNA obtained elsewhere. STR profiling is useful in many fields, including forensic science, paternity testing, bone marrow transplant monitoring, cell line authentication, and linkage mapping. A956 [Struhl Report] ¶ 5; A979 [Booker Report] ¶ 5.

STR analysis requires the making of copies of the STR loci of interest to obtain a detectable amount for analysis. A956-957 [Struhl Report] ¶ 6; A979-980 [Booker Report] ¶ 6. This copying process is referred to as "amplification." For efficiency reasons, it is valuable to co-amplify several loci in a single reaction rather than each individually. A979-980.

There are several steps in the amplification process for STR loci. First, one separates double-stranded DNA into single strands. Then one introduces "primers," which are known to target the desired STR locus. These primers bind to the single strands near the target loci and effectively mark the ends of the locus to be copied—like book-ends encompassing the DNA region of interest. A pair of primers is used for each locus—one to mark each end of the strand. So long as each primer in a pair is far enough outside the specific STR region so that the area of interest remains between the "bookends" it is a candidate for use. Accordingly, there are countless primer combinations that can be selected for any locus. A363 ['771 Patent] at 12:39-41 ("It is understood that many different sets of primers may

be developed to amplify a particular set of loci."). The single strands of DNA can be replicated beginning at the primer sites into double-stranded DNA. Finally, the process is repeated until a sufficient number of copies of the desired STR loci are generated. A979-980 [Booker Report] ¶ 6. This process is referred to generally as a polymerase chain reaction (PCR), and when it involves the copying of multiple STR loci at the same time, it is called multiplex PCR amplification. Because multiplex PCR amplification simultaneously targets multiple STR loci, it requires the use of multiple primer pairs (one pair for each locus) in a single reaction. A979-980.

A single allele of a single locus is generally not, by itself, sufficiently unique to an individual to establish with confidence that a match means that the two samples came from the same person. A1378-1379 [Defs.' Responses to Findings of Fact] ¶¶ 6-7. Instead, alleles at multiple loci must be compared to obtain a statistically significant comparison. A1378-79. The more loci compared the less the chance that two samples will result in a false match. A1378-1379.

B. THE UNPREDICTABILITY OF WHETHER A GIVEN SET OF LOCI WILL SUCCESSFULLY CO-AMPLIFY

Promega did not invent multiplex PCR amplification of STR loci. A937, A939-940 [Struhl Invalidity Report] ¶¶ 5, 12; A1048-1055 [Chamberlain, *Deletion Screening*]; A1057-1066 [Chamberlain, *Multiplex PCR*]; A1370 [Promega's Responses to Proposed Findings of Fact] ¶ 20 (undisputed); A939-940 [Struhl

Invalidity Report] ¶ 12; A1068-1077 [Kimpton '94]; A1079-1088 [Kimpton '93]; A1090-1038 [Caskey]; A1129-1139 [Edwards]; A1370-1371 [Promega's Responses to Proposed Findings of Fact] ¶ 22 (undisputed). The concept of multiplex amplification reactions dates back to the late 1980s. A1370 ¶ 21 (undisputed). By the early 1990s, scientists were successfully carrying out multiplex reactions of new and different sets of STR loci. A1068-1077 [Kimpton '94]; A1079-1088 [Kimpton '93]; A1090-1128 [Caskey]; A1129-1139 [Edwards].

Despite its widespread use, multiplex PCR remained an unpredictable method. A938 [Struhl Invalidity Report] ¶ 7; A1371 [Promega's Responses to Proposed Findings of Fact] ¶ 24 (undisputed); A678 [Promega Brief re: SJ] (Promega stating that "there is no dispute regarding the unpredictability of STR multiplexing."). Scientists discovered that co-amplifying multiple loci together was more complicated than simply consolidating multiple individual amplification reactions into one. *Id.*; A1371 [Promega's Responses to Proposed Findings of Fact] ¶ 25 (undisputed). That is, even if scientists know how to amplify one particular locus and also how to amplify another locus, they did not therefore know how to perform a multiplex PCR that would co-amplify both of those same two loci in the *same* mixture. A1371-1372 ¶¶ 25-30 (undisputed). Multiplexing could produce unanticipated artifacts and introduce challenges that did not arise when performing single locus amplification. *Id.*; A1371 ¶ 26 (undisputed). Multiplex

PCR for a particular set of loci required its own trial and error tests to confirm that it would work, even if the amplification process for each individual locus in that set were known. A1372 ¶¶ 30-31 (undisputed).

Moreover, even as scientists undertook the trial and error efforts to determine a reliable multiplex PCR amplification process for particular sets of loci, they did not uncover an alternative to the trial and error process each time amplification of a new set of loci was sought. A1368, 1374-75 ¶¶ 8, 9, 42; A1371-72 ¶¶ 27 (undisputed), 28 (undisputed), 30 (undisputed). Scientists could not predict with any certainty, absent a preexisting publication or teaching, whether a given set of loci would co-amplify successfully together using particular primer pairs. A1368 ¶ 8, A1372 ¶ 29 (undisputed).

Experimentation, through trial and error, was required even when adding a new locus to an already successful multiplex. A1368 ¶ 9; A1372 ¶¶ 30-31 (undisputed). Scientists had no way (except through trial and error) to know how the new locus would interact with the known set or how the primers needed for the new locus would work in a reaction involving the known set of loci and their known primers: indeed an entirely new set of primers for all loci might have to be tested. A1368 ¶ 9, A1372-73 ¶¶ 30-33, 35 (undisputed).

Moreover, the difficulty of successfully performing multiplex PCR amplification only increased with the addition of each new locus. Adding a third

loci to a known set of two involved a certain level of complication, but adding a seventh to a known set of six involved even greater complexity, and adding an eighth locus to a known set of seven involved still greater complexity. *Id.*; A1143-44 [Gibbs Report]; A1372 [Promega's Responses to Proposed Findings of Fact] ¶ 32 (undisputed).

The problems that may arise when adding loci are many. But the potential conflicts among primers merit special attention for this appeal. Primers are the crucial component to make any multiplex reaction work because conflicts among primers are unpredictable and intolerable. A939 [Struhl Invalidity Report] ¶ 10; A1373 [Promega's Response to Proposed Findings of Fact] ¶ 34. If a particular multiplex amplification fails because the primer pairs would not work together, new primer candidates have to be designed and tested potentially for all loci in the mixture. *Id.*; A1373 [Promega's Response to Proposed Findings of Fact] ¶ 35 (undisputed). The identification of primer pairs for each locus that would work together in a multiplex was described by Promega during prosecution as "unpredictable," "laborious," and a "difficult, arduous process"—even if only one new locus is being added to a small set. See A1238-1240 [Promega's Statements During Prosecution].

Over time skilled artisans managed to co-amplify particular sets of loci through laborious trial and error. For example, the early Caskey patent describes a

multiplex reaction of two particular STR loci. A1112-1116 [Caskey '759] at 15-16 (Example 5) and Fig. 3. The Kimpton '93 reference describes a particular 3-plex, 4-plex, and also a particular 7-plex. A1080-1081 [Kimpton '93] at 14-15. By 1995, a "highly discriminating octoplex [8-plex]" had been achieved, as explicitly acknowledged by the '660 patent. A227 [Patent '660] at 3:1-10. Finally, by the time of the '235 and '771 patents, numerous multiplex systems "containing up to 11 separate STR loci ha[d] been described." A311 [Patent '235] at 3:5-23 (emphasis added); A358 [Patent '771] at 3:10-28 (emphasis added).

The proliferation of particular successful multiplex PCR amplifications should not be confused with progress in predicting *whether* a new set of loci could be successfully co-amplified. As Ann Lins, an inventor of the '598 patent, and long-time Promega employee, explained the process was one of blind trial and error:





A1153-1155 [Lins Depo. Tr.] at 8:8-9, 18:20-21, 65:24-67:17.

III. THE ASSERTED PATENTS

The Promega patents, and the Tautz patent all relate to the multiplex amplification of STR loci. The claims recite a method or kit for simultaneously determining the alleles present in a set of STR loci from DNA samples, comprising: (a) obtaining a DNA sample; (b) selecting a set of loci of the DNA sample to amplify, including at least the specific loci recited in the claim; (c) coamplifying the selected loci in a multiplex amplification reaction; and (d) evaluating the amplified alleles to determine the alleles present at each loci, *i.e.* how many repeats are present at each loci. *See, e.g.*, A338, A340 ['235 Patent] Claims 1, 18.

The following table summarizes each patent. The complexity column summarizes the size of the disclosed sets of STR loci in the patent, e.g., 3-plex, 4-plex, 13-plex. The claim construction column summarizes whether the district

court construed the claims as "limited to products that use no loci other than those listed in the claims" (closed), or as not so limited (open-ended). A2372-2373 [Order].

Patent	Filing	Asserted Claims	Complexity	Claim
	Date			Construction
' 660	1996	2-5, 16-17, 19-21, 23-	3-8	Closed as to
		25, 27-31		claims 25, 27-
				31; open as to
				remaining claims
' 235	1998	1-4, 6-13, 15-19, 21-23	13	Open
' 598	1999	1-2, 4-5, 7-10, 12, 15,	3	Open
		19, 21-24, 27-28, 31-33		
'771	2002	5	14	Open
' 984	1993,	15-16, 18, 23, 25, 27-	n/a	n/a
(Tautz)	2000	28, 41-42		
	(reissue)			

To obtain the Promega patents, Promega relied upon the alleged novelty of the specific loci combinations recited in the claims and the primers that were found to work together in that multiplex without conflict. For example, in prosecuting the parent application to the Promega patents, the applicant stated that "the prior art simply cannot provide any indication of which parameters are critical, nor can the prior art provide any direction as to which of many possible choices is likely to be successful because the cited references admittedly do not teach the claimed loci combinations." A996 [U.S. Application 08/316544 File History] (emphasis in original), A997 (applicant stating that the prior art references "do not disclose or suggest that any arbitrary combination of loci can be co-amplified without undue

experimentation."); A1012 ['660 Patent File History] ("Applicants submit that none of the cited references discloses the methods for selecting, co-amplifying, and evaluating the specific sets of short tandem repeat loci selected and analyzed" in the claims); see also A1360 [Promega's Opp. to Mot. for Partial SJ] ("The lack of these novel and unobvious locus combinations in the prior art, together with the unpredictable nature of this art, is fatal to Defendants' obviousness arguments."); A1357 ("The specific combinations of loci in the asserted claims of the '598 Patent are unique to the '598 Patent."); A1359-1362 ("Importantly, these changes [in reaction conditions] necessary for adding the locus were determined empirically and the final successful conditions could not be predicted. . . . Given this lack of predictability, these multiplexes are not obvious."); A1363 (stating that the Promega patents do not "broadly claim co-amplification of 'STR loci' [but] instead [claim] the specific combination(s) of loci set forth in each asserted claim."). Seventy-one such statements from the prosecution histories are collected at A1223-1231 [Promega's Statements During Prosecution]; see also A227 ['660 Patent] at 3:46-61 ("Multiplex analysis of the sets of specific STR loci disclosed herein have not been previously described in the prior art."). It is undisputed that the alleged novelty of each Promega patent resides in the specific combinations of loci set forth in each asserted claim.

To illustrate the alleged novelty of Promega's claims, consider the following table. The table provides examples of sets of loci described in the prior art as suitable for multiplex amplification:

Prior Art Loci Set	Promega Loci Set	Difference
HUMCD4,	HUMCD4,	'598 claim 1 substitutes
HUMARA,	HUMCSF1PO,	one locus
HUMTH01 ⁶	HUMTH01	
HUMVWFA31,	HUMVWFA31,	'598 claim 1 removes two
HUMTH01,	HUMTH01,	loci and adds one
HUMF13A1,	HUMCSF1PO	
HUMFES/FPS ⁷		
HUMEFSFPS,	D3S1539,	'660 claim 1 substitutes
HUMTH01,	HUMTH01,	one locus
HUMF13A01,	HUMF13A01,	
HUMVWFA31 ⁸	HUMVWFA31	

IV. THE ACCUSED PRODUCTS

The accused products are the following Life kits for genetic testing: AmpFlSTRTM Profiler Plus, AmpFlSTRTM COFiler, AmpFlSTRTM Profiler, AmpFlSTRTM Identifiler, AmpFlSTRTM Yfiler. A416, A418-420, A421, A423

⁶ See A1035 ['598 Patent File History (Fregeau)].

⁷ See A1030 ['598 Patent File History (Kimpton)].

⁸ See A1012-1015 ['660 Patent File History] (Urquhart and Kimpton-I) ("Applicants respectfully submit that the method of claim 1, as amended herein to remove one of the four loci cited in Urquhart et al. (i.e. removing the HUMFESFPS locus), is clearly not anticipated . . . that reference fails to disclose the selection of sets of loci for multiplex analysis which include at least four loci selected from the list of loci in claim 1, as amended herein.").

[Complaint] ¶¶ 27, 36, 45, 54, 65. Each kit provides components for carrying out the multiplex amplification of STR loci from DNA samples. The kits contain a number of components stored in tubes, including: a primer mix, a PCR reaction mix, a DNA polymerase (PCR) enzyme, and a control DNA from another human source. A2049 [2/7/2012 Trial Tr.] at 50:17-52:12; *see also* A2302 [Defs. Brief in Support of Rule 50(b) Mot.] (citing '984 patent claim 42). The kits are assembled in Warrington, United Kingdom, and sold worldwide. A2265-2266 [2/13/2012 Trial Tr.] at 40:24-41:7.

The Identifiler kit amplifies a total of 16 STR loci. The Profiler kit amplifies ten. The COfiler kit amplifies seven. Each of these kits amplifies STR loci beyond the loci specified in the asserted claims of the Promega patents. See A932 [AmpFlSTR® Kit Product Portfolio]; A1233-1236 [Loci Amplified by the AmpFlSTR® Kits]. For example, the '660 patent claims specify sets that include 3 to 8 loci. The Identifiler kit amplifies between 8 and 13 more loci than those specified in the '660 patent claims. A1233-1234 [Loci Amplified by the AmpFlSTR® Kits].

⁹ The kits may also contain mineral oil and allelic ladders, which are control sets of DNA run on the gel next to a sample.

¹⁰ The Profiler Plus and Yfiler kits were only alleged to infringe the Tautz patent.

V. THE 2006 CROSS LICENSE

The parties' 2006 Cross License grants defendant Applied Biosystems a non-exclusive license under the Promega patents and the Tautz patent for "Forensics and Human Identity Applications," which is defined as:



See A816, A819 [2006 Cross License] §§ 1.6, 1.7, 2.1.2; see also A1866, A1868, A2004, A2007 [2/7/2012 Trial Tr.] at 61:9-19, 63:3-11, 5:11-25, 8:15-18 (background on the license agreement). At a February 6, 2012 oral hearing, the district court ruled that "Forensics and Human Identity Applications" is limited to use in a live forensic investigation conducted by police officers. See A1792 [2/6/2012 Trial Tr.] at 19:1-4 ("I think it's just obvious that research, educational and training is not covered by the [2006] license unless it's done right there in the police department."). Based on this ruling, Promega attempted at trial to collect damages for kits sold to numerous organizations that carry out forensics research, education, and training. This includes, for instance, numerous universities with accredited forensic training programs. See, e.g., A1572 [Declaration of G.

Sandulli] ¶ 39. Life appeals the district court's February 6, 2012 oral ruling regarding the scope of the 2006 Cross License.

SUMMARY OF THE ARGUMENT

Promega has been asserting its patents against Life over the course of a decade. The Promega patents are invalid and the commercial playing field should be cleared of them because they impede legitimate competition.

First, Promega has included in its patents overbroad claims via the tactic of open-ended claiming at the point of novelty. This Court in *Magsil* recently confirmed that open-ended claim elements must be enabled to their full scope, especially where they are open-ended at the point of novelty. Yet, contrary to that ruling, the district court in this case concluded that the "unrecited" loci in the sets included in the open-ended claim elements of the Promega patents need not be enabled.

A comparison between the overbroad claims and limited patent disclosure makes clear that they are not commensurate. The patents only enable *particular* disclosed STR loci multiplex sets when using *particular* primers. But the openended claim elements purport to cover multiplex sets that include any number of STR loci and any primer pairs—far beyond those recited or disclosed. Even Promega cannot contend such multiplex sets or primer combinations are enabled. Indeed, Promega strenuously argued below and to the PTO that it is impossible to

adding a *single* new locus to an already successful multiplex. Yet, Promega attempts to claim all such unpredicted and unpredictable multiplex sets as its invention. The claims are invalid because they are not enabled to the full scope of the open-ended multiplex sets they cover.

Second, the Promega patents are obvious. It is undisputed that the working multiplex sets disclosed in the patents and the multiplex sets expressly recited in the claims are the product of rote trial and error without the benefit of any shortcuts or other generalized innovations. The STR loci and primers that were found to work and that are expressly recited in the claims are not special beyond the fact that trial and error revealed that they can be multiplexed together. This is not inventive activity. Accordingly, the claims are obvious because the smallest, expressly recited multiplexes they cover are marginal products of the mere practice of "trial and error." This is true even though the claims also are not enabled because they cover countless multiplexes that the patents fail to disclose and certainly do not teach how to successfully co-amplify.

Finally, the district court misinterpreted Life's 2006 license to the patents in an important way. The district court incorrectly concluded—based on little analysis of the evidence in the record—that "it's just obvious that [forensic] research, educational and training is not covered by the license unless it's done

right there in the police department." A1792 [2/6/2012 Trial Tr.] at 19:1-4. This interpretation of the 2006 Cross License is overly narrow and inconsistent with the plain language and overall purpose of the license.

The court's interpretation is at odds with the plain language of the license grant, which contemplates that licensed uses include not just those that are performed by the police or in a police department, but more broadly that are necessary for use in legal proceedings and uses that are "in preparation for" legal proceedings. At a minimum, the license grant is ambiguous and consideration of extrinsic evidence is warranted. The evidence presented to the district court established that forensic research, training, and education applications of specific STR kits are mandatory gateway applications that must be carried out before any specific STR kit (including Life's STR kits) can actually be used in the field by forensic scientists. Numerous standards bodies (e.g., the FBI and Forensic Science Education Programs Accreditation Commission) mandate that forensic scientists be educated and trained regarding the kits that they will ultimately use in casework, and such training, even if done at a university, is thus "in preparation for" legal proceedings and within the scope of the 2006 Cross License. Unless defendants are permitted to sell their STR kits for these applications, forensic scientists cannot be trained and qualified to use them, and the market for them will diminish. Thus, under the district court's interpretation, defendants would be

unable to sell freely STR kits for use in or preparation for legal proceedings and would not receive the benefit of the bargain they struck in the 2006 Cross License. The court's interpretation is thus facially unreasonable and cannot be adopted.

STANDARD OF REVIEW

Summary judgment grants are reviewed without deference by this Court under the Seventh Circuit's *de novo* standard. *See Dempsey v. Atchison, Topeka and Santa Fe Ry. Co.*, 16 F. 3d 832, 836 (7th Cir. 1994). Summary judgment in favor of Promega would have been proper only where, "viewing the record and all reasonable inferences drawn from it in the light most favorable to the nonmovant," there are no disputed issues of material fact. *Dempsey*, 16 F. 3d at 836.

Life's appeal presents two patent issues: enablement and obviousness. Both of those invalidity grounds must be proven by clear and convincing evidence and are legal questions based on the factual record. *Magsil Corp. v. Hitachi Global Storage Techs., Inc.*, 687 F.3d 1377, 1380 (Fed. Cir. 2012); *KSR Intern. Co. v. Teleflex Inc.*, 127 S. Ct. 1727, 1745-46 (2007).

The license issues on appeal are governed by California law pursuant to a choice of law clause in the 2006 Cross License. A827 [2006 Cross License] § 7.5. Under California law, interpretation of a contract, including whether contract language is ambiguous, is a judicial function reviewed *de novo*. *Cachil Dehe Band of Wintun Indians v. California*, 618 F.3d 1066, 1073, 1075 (9th Cir. 2010).

Under California law, "when there is a material conflict in extrinsic evidence supporting competing interpretations of ambiguous contract language the court may not use the evidence to interpret the contract as a matter of law, but must instead render the evidence to the factfinder for evaluation of its credibility." *Id.* at 1077.

ARGUMENT

I. THE DISTRICT COURT SHOULD HAVE GRANTED SUMMARY JUDGMENT IN FAVOR OF LIFE, NOT AGAINST IT, BECAUSE THE OPEN-ENDED CLAIMS ARE NOT ENABLED

The claims of the Promega Patents that cover limitless sets of multiplexes and primers are invalid because they are not enabled. The district court nevertheless granted summary judgment extinguishing Life's declaratory judgment counterclaim. But the district court never compared the broad scope of the claims to the limited teachings of the patents. Nor did it acknowledge the unpredictability of developing the wide-ranging multiplex sets of loci encompassed by the claims, but not taught by the patents.

The open-ended claims of the Promega patents asserted in this case are: claims 2-5, 16-17, 19-21, 23-24 of the '660 patent (A257-260); claims 1-4, 6-13, 15-19, 21-23 of the '235 patent (A338-340); claims 1-2, 4-5, 7-10, 12, 15, 19, 21-24, 27-28, and 31-33 of the '598 patent (A288-291); and claim 5 of the '771 patent (A387).

Instead of confronting the substance of the enablement analysis, the district court merely accepted Promega's argument that multiplex sets that include "unrecited elements" do not need to be enabled at all. A28 [Order] ("In the absence of case law requiring the patentee to enable his invention with respect to unrecited elements, I decline to impose such a requirement."). This mistaken approach resulted in the wrong outcome.

Applying this Court's precedent to the undisputed factual record confirms that the claims of the Promega patents are, indeed, not enabled because they are vastly overbroad.

A. BROAD CLAIMS MUST BE ENABLED ACROSS THE FULL SCOPE OF THEIR COVERAGE

It is a bedrock principle of patent law that the full scope of patent claims must be enabled by the teachings of the patent. *Magsil Corp. v. Hitachi Global Storage Techs.*, *Inc.*, 687 F.3d 1377, 1380-81 (Fed. Cir. 2012).

In *Magsil* this Court recently explained the importance of this time-tested safeguard against over-claiming:

Enablement serves the dual function in the patent system of ensuring adequate disclosure of the claimed invention and of preventing claims broader than the disclosed invention. This important doctrine prevents both inadequate disclosure of an invention and overbroad claiming that might otherwise attempt to cover more than was actually invented. Thus, a patentee chooses broad claim language at the peril of losing any claim that cannot be enabled across its full scope of coverage.

(citation omitted); see also Sitrick v. Dreamworks, LLC, 516 F.3d 993, 999 (Fed. Cir. 2008) ("The scope of the claims must be less than or equal to the scope of the enablement to ensure that the public knowledge is enriched by the patent specification to a degree at least commensurate with the scope of the claims."); Wyeth and Cordis Corp. v. Abbott Labs., No, 2012-1223, Slip Op. at 5 (Fed. Cir. June 26, 2013) ("Claims are not enabled when, at the effective filing date of the patent, one of ordinary skill in the art could not practice their full scope without undue experimentation.").

The enablement analysis at its core requires a comparison of the scope of the claims with the scope of the disclosure. *Magsil*, 687 F.3d at 1381 ("The specification must contain sufficient disclosure to enable an ordinarily skilled artisan to make and use the entire scope of the claimed invention at the time of filing."). Open-ended claim elements are subject to full scrutiny for lack of enablement and require disclosures commensurate with their broad scope. *Id.* at 1383 ("The open claim language chosen by the inventors does *not* grant them any forgiveness on the scope of required enablement.").

B. THE OPEN-ENDED CLAIMS OF THE PROMEGA PATENTS ARE BROAD

A central dispute in the district court was whether the sets of loci claimed in the patents are "open-ended" or "closed." Because the multiplex sets of the accused kits include numerous loci beyond those taught in the Promega patents,

Promega insisted that all its claims were open-ended in describing the loci in the claimed multiplex "sets." Thus, for example, it argues that Claim 12 of the '598 Patent, which identifies only a combination of three loci for the claimed set, covers a product that is a 9-plex set. *See, e.g.*, A878 [Declaration of R. Dimond] ¶ 117; A1284 [Promega's Proposed Findings of Fact] ¶ 188.

A handful of non-infringed claims (Claims 25 and 27-31 of the '660 patent) were construed to have closed multiplex "sets." But most of the claims were construed as covering open-ended sets and thus encompass multiplex sets with a *limitless* number and combination of loci so long as they include the listed loci. ¹² *See* A21-22 [Order]. Further, the asserted claims are *not* limited to the primer sets disclosed in the patents. Rather they cover multiplex sets regardless of whether the primers Promega disclosed as successful in the Promega patents are used—or not.

The Promega patents do include some claims limited to the sets of loci and primers actually disclosed in those patents. But those claims are not infringed by Life's products. Thus, the open-ended claims are the heart of this case.

¹² The claims construed to have "closed" sets were found not infringed. *See* A21-22 [Order].

C. THE DISCLOSURE IN THE PROMEGA PATENTS IS NARROW, DOES NOT REDUCE THE UNPREDICTABILITY OF CREATING NEW MULTIPLEXES, AND IS NOT COMMENSURATE WITH THE CLAIM SCOPE

While the open-ended claims of the Promega patents are broad, the disclosure in the Promega patents is strikingly stingy. The patents disclose as their inventions particular multiplex sets that work using only particular primers.

As explained above, a multiplex is comprised of multiple loci from a DNA sample that can be amplified together so long as the selected primers do not conflict with each other. As a general rule, the more loci used in a multiplex, the better the accuracy of identifying a unique DNA sample because there are more data points, but also the more likely that problems arise such as conflicts between the primer pairs needed for each locus.

The multiplexes described in the Promega patents as the invention include loci used in prior art multiplexes. For example, Claim 1 of the '598 Patent, claims a 3-plex that includes two loci from a prior art 3-plex and adds a third locus. *Compare* A1035 ('598 patent file history admitting that "Fregeau *et al.*... identif[ies] two sets of loci that could be co-amplified in separate multiplex amplification reactions, specifically . . . (2) HUMCD4, HUMARA, and HUMTH01.") *with* '598 patent, claim 1 (A228-289) (claiming "(b) selecting a set of at least three short tandem repeat loci . . . selected from the group consisting of: HUMPOX, HUMTH01, and HUMCD4"). For this 3-plex, the disclosure describes

only *one* possible primer pair for each locus that can be successfully used. *See* A289 ['598 Patent] Claim 3.

Promega's position in obtaining and defending the Promega patents, during prosecution and in litigation, is that the creation of a new multiplex, even if it includes prior art loci combinations, is a new invention because it is always unpredictable whether a new multiplex will work. A682 [Promega Brief in Support of SJ] ("any set of STR loci, including all those in the Promega Patents, is special in the sense that one cannot predict that they can ever be multiplexed prior to inventing conditions where their co-amplification is, in fact, successful.").

According to Promega, the creation of any new multiplex is a matter of "trial and error." A678-679 [Promega Brief in Support of SJ]; A1387 [Defs.' Responses to Proposed Findings of Fact] ¶ 36 ("Combinations of loci that can be successfully multiplexed are generated by trial and error of locus combinations and by adjustment of primer concentrations to identify an equilibrium in which all included loci may be amplified."). There were no insights or "short cuts" that Promega discovered for selecting loci that could work together in a multiplex—or to help identify primers that do not conflict. A1153-1155 [Lins Depo. Tr.] at 8:8-9, 18:20-21, 65:24-67:17.

Promega inventor Ann Lins, who worked on multiplexing at Promega in the key 1992 to 1998 era, explained this vividly. She testified that, even with all their

work and supposed inventions, the inventorship team's inability to predict whether a multiplex set would work before testing *never* improved over the years. A1153-1155 [Lins Depo. Tr.] at 8:8-9, 18:20-21, 65:24-67:17. The creation of new multiplex sets was as unpredictable before the Promega patents as it was afterwards. The disclosure is that narrow.

Understanding why creating a successful new multiplex was unpredictable, even if it included merely adding one new loci to existing multiplexes, requires an appreciation of the undisputedly important role of primers. A1373 [Promega Responses to Proposed Findings of Fact] ¶ 34 (undisputed). A key aspect of multiplexing loci is ensuring that the primers used for one locus do not interfere or conflict with primers used for another. A715-716 [Declaration of J. Ballantynel ¶ 11 (Promega's expert stating that "[o]ne of the most significant problems in adding new loci is unpredictable interactions between primers."); A678 [Promega Brief in Support of SJ]; A678 [Promega Brief in Support of SJ] ("the wrong primers can create serious STR multiplexing problems. . . . Inappropriate selection of primers can produce several undesirable effects"). There are countless primer combinations that can be tried for any locus. A363 ['771 Patent] at 12:39-41 ("It is understood that many different sets of primers may be developed to amplify a particular set of loci.").

Whether one set of primers for one locus can be made to work with another set of primers for a different locus was, by all accounts, unpredictable. A678 [Promega's Brief in Support of SJ] (Promega stating that "there is no dispute regarding the unpredictability of STR multiplexing."); A1371-1372 [Promega's Responses to Proposed Findings of Fact] ¶¶ 24-31 (undisputed). Thus, if one attempts to build on a prior art two-loci amplification (a "2-plex"), the primers used for each of the loci in a larger multiplex (e.g., three loci, or "3-plex") may well have to be developed from scratch without any benefit from the success of the prior art 2-plex. What is ultimately required is that the primers tested for all of the loci in a multiplex set work together, which is an unpredictable question that can only be answered by trial and error. For that reason, each new multiplex is its own new interrelated "set" of loci requiring its own trial and error testing that may or may not ultimately be able to employ any of the primers used in successful prior art multiplexes that share a subset of loci.

The only passage in the Promega patents that Promega identified in the district court as reflecting some kind of "novel aspect" teaching in multiplex development merely underlines the problems that occur if the wrong primers are selected:

Inappropriate selection of primers can produce several undesirable effects such as lack of amplification, amplification at multiple sites, primer dimer formation, undesirable interaction of primer sequences from different loci, production of alleles from one locus which

overlap with alleles from another, or the need for amplification conditions or protocols for the different loci which are incompatible in a multiplex.

A1361 [Promega's Opp. to Mtn. for Partial SJ] (quoting '598 patent at 7:4-11). This passage adds nothing to advance the development of new multiplexes, leaving that process fraught with unpredictability and without short-cuts. A1362 ("Importantly, these changes necessary for adding the locus were determined empirically and the final successful conditions could not be predicted."). The patent thus leaves those skilled in the art without any guidance for developing a new multiplex beyond those expressly recited in the patent; those skilled in the art have merely been shown a place to start as they try to develop multiplexes beyond those expressly recited in the patent. Simply put, Promega did not arrive at—or disclose—any novel teaching about how to develop multiplexes or select suitable primers that was not already known in the prior art.

This Court's recent decision in *Wyeth and Cordis Corp. v. Abbott Labs.*, No, 2012-1223 (Fed. Cir. June 26, 2013), makes clear that merely providing a starting point for unpredictable trial and error is not enablement. There, patents were held invalid as a matter of law due to overbreadth. The claims covered some enabled embodiments, but also covered thousands of other embodiments that were not taught by the patent. The patentee argued that the full scope was enabled because persons skilled in the art, through basic trial and error experimentation, could

discover all the embodiments that worked. This Court explained that even if the full scope of a claim can be achieved through supposedly "routine" trial and error experimentation, the claims are not enabled where the specification "discloses only a starting point for further iterative research in an unpredictable and poorly understood field." *Wyeth*, Slip. Op. at 10.

Wyeth covers this case like a glove. Here, the full scope of the claim covers thousands and thousands of multiplex sets and countless primer combinations for the disclosed multiplexes. Yet the indisputable evidence, including from Promega's own witnesses, establishes that the trial and error experimentation required to achieve the full breadth of the claim would be unpredictable and require a massive effort.

In short, the disclosure of the Promega Patents is not commensurate with the scope of the claims. While these patents disclose particular multiplex sets of loci, and particular primers that make them work, they teach nothing that would enable persons skilled in the art to make the vast number of loci and primer combinations within the scope of the broad claims. Because the inclusion of each additional locus represents not a linear or merely additive effort but rather an exponential increase in effort, it is grossly disproportionate to allow the discovery of particular primer combinations that work for a 3-plex to capture products that, thanks to

arduous additional efforts, use 16- or 20- or 100-plex combinations. The Promega patents have not at all contributed to the development of such products.

D. THE DISTRICT COURT'S "UNRECITED ELEMENTS" ANALYSIS IS FLAWED

The district court's construction of the claims at issue as "open-ended" means they encompass an unlimited number of multiplex sets enabled by an unlimited number of primer pairs for each locus. The district court's rejection of Life's enablement proof essentially started and ended with its conclusion that the unlisted loci in these infinite multiplex sets were "unrecited elements" exempt from an enablement analysis. A27-28 [Order]. The district court did not cite any enablement case to support its "unrecited element" exemption and none supports its holding.

The district court was wrong on both the facts and the law. The district court's conclusion that the unlisted loci are merely "unrecited elements" of the accused product that need not be enabled was erroneous. This is not a situation where an accused product includes each element of the claimed invention and extraneous structures or functions that are not recited in the claims. Rather, the claims here expressly recite, as an affirmatively stated *part of the claim*, a set of STR loci that is to be multiplexed, or at least a mixture that is the product of the

multiplex amplification reaction.¹³ As construed by the district court, those claimed sets of STR loci and/or the claimed multiplex mixture include both the particular loci recited in the claim *and* all *unlisted* STR loci that might be used in a multiplex set. In other words, the unlisted loci are part of the claimed set and/or claimed multiplex mixture. And, the unlisted loci are not extraneous; they profoundly affect the reaction's chemistry.

The district court's "unrecited element" analysis also conflicts squarely with this Court's enablement analysis in *Magsil*. In that case, the patentee contended that its claim was merely a standard "open-ended" claim and that it was therefore not necessary to enable the full scope of potential accused devices that would be

¹³ For each of the "open-ended" claims at issue, the district court generally construed the claim element of a "set of . . . loci" to be open-ended. A8-21 [Order]. The district court arrived at a similar conclusion via a different analysis for claims 2-5 and 16 of the '660 patent, and the asserted claims of the '660 patent dependent on claim 16. A13-17. For those claims, the district court concluded that "set of loci" in the pertinent independent claim was open based on analysis of the dependent claims and prosecution history. See A16; see also A664, A672 (Promega arguing that the set of loci in claims 2-5 and 16 "encompass" unlisted loci and that claimed multiplex reaction is not limited to only listed loci, but instead the listed loci "establish the minimum size of the multiplex"). The court also concluded, based on Promega's argument, that the multiplex "mixture" in step (c) of the claims was an open-ended claim element that included the products of multiplexing unlisted loci. See A15. Regardless of whether the "set of loci" or the "mixture" is open, each of the claims as construed by the district court affirmatively includes a broad, open-ended element that must be enabled. Life preserves its objections to all these constructions.

encompassed by the claim. This Court rejected that argument: "The open claim language chosen by the inventors does not grant them any forgiveness on the scope of required enablement." *Magsil*, 687 F.3d at 1383. Thus, this Court compared the narrow scope of the teaching in the patent to the broad scope of the claim, *including* its open-ended elements. *Id.* It determined that they were not commensurate and that the patent was thus invalid as a matter of law.

In sum, Promega did not enable the full scope of its claims under the claim construction successfully argued by Promega. The Promega patents teach far too little to be commensurate with the broad claim scope. Accordingly, the district court's grant of Promega's motion for summary judgment of enablement should be vacated, and Life's motion for summary judgment of non-enablement should be granted as a matter of law.¹⁴

II. THE DISTRICT COURT SHOULD HAVE GRANTED SUMMARY JUDGMENT IN FAVOR OF LIFE, NOT AGAINST IT, BECAUSE THE CLAIMS OF THE PROMEGA PATENTS ARE OBVIOUS

The asserted claims of the Promega Patents are invalid because they are obvious. 15 The district court nevertheless granted summary judgment

¹⁴ Application of the *Wands* factors is not required, as illustrated by *Magsil*. However, the result is the same using that framework. Those factors are essentially all considered in this brief.

¹⁵ The asserted claims of the Promega patents are: claims 2-5, 16-17, 19-21, 23-25, 27-31 of the '660 patent (A257-260); claims 1-4, 6-13, 15-19, 21-23 of the '235

extinguishing Life's declaratory judgment invalidity counterclaim. That ruling should be reversed and Life's motion for summary judgment of obviousness should be granted as a matter of law.

A. THE CLAIMS OF THE PROMEGA PATENTS ARE OBVIOUS

Obviousness is governed by the familiar four factor framework of *Graham* v. *John Deere Co.*, 383 U.S. 1, 17 (1966): (1) the scope and content of the prior art, (2) the level of ordinary skill in the art, (3) the differences between the claimed invention and the prior art, and (4) objective indicia of non-obviousness.

Here, the factual record establishing obviousness is undisputed:

- The prior art references relied upon by Life (Kimpton and Caskey) show the successful use of multiplex STR loci for DNA identification purposes. ¹⁶
- The prior art literature extensively describes multiplex amplifications and the benefits of multiplexing. ¹⁷

patent (A338-340); claims 1-2, 4-5, 7-10, 12, 15, 19, 21-24, 27-28, and 31-33 of the '598 patent (A288-291); and claim 5 of the '771 patent (A387).

¹⁶ A1370-1371 [Promega Responses to Proposed Findings of Fact] ¶¶ 22-23 (undisputed); A939-940 [Struhl Invalidity Report] ¶ 12; A1068-1077 [Kimpton '94]; A1079-1088 [Kimpton '93]; A1090-1127 [Caskey]; A1129-1139[Edwards]; A1030 [Kimpton]; A984 [Caskey].

¹⁷ See A226 ['660 Patent] at 2:3-3:13 (stating in Background of the Invention that "Polymorphic STR loci are extremely useful markers. . . Such 'multiplex' amplifications, as they are called, have been described extensively in the literature. Multiplex amplification sets have been extensively developed . . ."); A1223-1231 [Promega's Statements During Prosecution].

- The patents acknowledge the desirability of using larger sets of STR loci for more precise human identification purposes.¹⁸
- The only difference between the claims and the prior art is the identity of the particular loci included in the multiplex. 19
- The selection of loci and primers for a multiplex is a matter of trial and error without short cuts.²⁰
- The successful selection of loci and primers was not predictable.²¹

¹⁸ See A229 ['660 Patent] at 8:14-17 ("Scientists, particularly forensic scientists, have long appreciated the need to analyze multiple polymorphic loci of DNA in order to ensure that a match between two samples of tissue is statistically significant.").

¹⁹ A675-76, 684 [Promega Brief in Support of SJ] (Promega stating that "[t]he asserted claims of the '598 patent specify different and unique triplexes and quadruplexes that are not described in the Caskey Patent. . . . The '235 and '771 Patents describe still larger and unique multiplexes not found in the Caskey Patent. . . . The asserted claims of the '598 patent specify different and unique triplexes and quadruplexes that are not described in the Kimpton '93 Paper. . . ."); A1370-1371 [Promega Responses to Proposed Findings of Fact] ¶ 22; A1223-1231 [Promega's Statements During Prosecution]; A939-952 [Struhl Invalidity Report] ¶¶ 12-45.

A1153-1155 [Lins Depo. Tr.] at 8:8-9, 18:20-21, 65:24-67:17; A1372 [Promega's Responses to Proposed Findings of Fact] \P 28, 30 (undisputed); A678 [Promega Brief in Support of SJ].

A1371-1372 [Promega's Responses to Proposed Findings of Fact] ¶¶ 24-25 ("Not Disputed that multiplex PCR was an unpredictable and experimental method at the time of the Promega Patents"), ¶¶ 28, 30 (undisputed); A682, A691 [Promega Brief in Support of SJ]; A938 [Struhl Invalidity Report] ¶ 7.

• Promega's inventorship team confirmed that the ability for one skilled in the art to predict whether a multiplex combination would work did not improve as a result of its work in this area.²²

This case presents an unusual record. The motivation to identify STR loci that co-amplify for DNA sample identification existed at the time of the claimed inventions. The task of trial and error testing of loci and primers in a multiplex is, however, a rote process based on known procedures. Indeed, the '598 Patent itself describes the discovery of new loci for a multiplex to be "in some sense, *routine* extensions of the core multiplex." A274 ['598 Patent] at 8:4-7.

The question presented by this issue is whether this kind of rote work, pursuing a well-defined prior art path, adds enough to the art to warrant a patent. As explained further below, this kind of trial and error in pursuit of a well-defined solution to a known problem is obvious and is unworthy of a patent.²³

In KSR Intern. Co. v. Teleflex Inc., 127 S.Ct. 1727, 1741 (2007), the Supreme Court explained that obviousness is not rigidly determined by formula. If the claimed subject matter is within the "ordinary skill" of a worker motivated to solve a problem, it is not an invention. Id. ("Granting patent protection to

²² A1153-1155 [Lins Depo. Tr.] at 8:8-9, 18:20-21, 65:24-67:17.

²³ While Life presented a claim-by-claim analysis at summary judgment, *see* A1202-1220, the legal question presented by this appeal cuts across all asserted claims of the Promega patents.

advances that would occur in the ordinary course without real innovation retards progress..."). Likewise, the Court explained that, if a person skilled in the art would have an "apparent reason to combine the known elements in the fashion claimed by the patent," that is indicative of obviousness. *Id.* at 1740-41. The Supreme Court concluded its opinion by explaining that "the results of ordinary innovation are not the subject of exclusive rights under the patent laws." *Id.* at 1746.

In light of these principles, applying the common sense obviousness standard of *KSR* to the facts of this case reveals that the claims are obvious. Having workers methodically test loci and primers via trial and error—without predictability or short cuts—to determine which multiplexes work and arrive at the multiplexes recited in the claims is not inventive. It is rote testing well within the ability of a person of ordinary skill in the art. There is no showing that the claimed loci have any special characteristics other than that trial and error happened to show that they work with particular primers in a particular multiplex set.

Importantly, an entire claim is invalid as obvious if the claim scope purports to cover *any* obvious advances over the prior art. *E.g.*, *In re Peterson*, 315 F.3d 1325, 1329–30 (Fed. Cir. 2003) ("we and our predecessor court have consistently held that even a slight overlap in range establishes a *prima facie* case of obviousness."). That is, an open-ended claim like that at issue here, might be

broad enough to purport to cover non-obvious advances over the prior art at one end of the scope, but also purport to cover only obvious improvements at the other end. Such claims, to be valid, must both enable the *full* scope of the invention out to the farthest reaches of claims beyond the prior art (which, as shown above, these patents do not) while also being sufficiently novel for even the smallest difference from the prior art to avoid obviousness. That is why the claims at issue here are both invalid as not enabled and obvious. Even though the level of experimentation required to enable the full scope of the claims is certainly undue, as explained above, and the Promega patents are thus not enabled, the rote testing to reach the multiplex sets expressly recited in the claims—i.e., the lower end of the claim scope—was well within the ability of those skilled in the art at the time of the invention.

The essence of Promega's response to this obviousness proof has been that whether any particular loci combination with particular primers can be multiplexed together successfully is totally unpredictable. *See* A1357-1358 [Promega's Opp. to Mtn. for Partial SJ].²⁴ But under *KSR*, there is no rigid formula that makes

²⁴ In its opposition to Life's motion for summary judgment of obviousness, Promega did not rely upon any alleged secondary considerations to support a finding of non-obviousness. *See* A1355-1362. In its motion for summary judgment of non-obviousness, Promega relied upon a single quotation from its expert expressing alleged skepticism regarding two of the Promega patents, the

unpredictability of results a guarantor of a valid patent. Where simple trial and error is reasonably expected to lead to a successful result, even if the particular STR loci that will work are unknown in advance, there may be diligence worthy of merit. The results of such diligence may warrant protection as a trade secret, but should not preclude others who likewise exercised the same diligence (or more) from enjoying the benefits of their hard work. This is particularly true because the STR loci that do work do not have any special characteristics other than that they have been found to co-amplify.

B. THE DISTRICT COURT'S SUMMARY JUDGMENT GRANT WAS ERRONEOUS

The district court set forth three reasons why it rejected Life's obviousness position. None supports summary judgment.

First, the district court found obviousness moot because it was supposedly "contingent on defendants' argument that the claims are not enabled." A2399 ("Because I have rejected defendants' enablement theory, this argument is moot."). This is confused. Life has argued that the patents may be *both* not enabled and obvious, but at no time has Life ever suggested that if the Promega patents are deemed enabled, they would also be not obvious. The analyses for enablement and

^{&#}x27;235 and '771 patents. A679. This single conclusory statement from Promega's expert is insufficient to overcome Life's showing of obviousness. *See Leapfrog Enterprises, Inc. v. Fisher-Price, Inc.*, 485 F.3d 1157, 1162 (Fed. Cir. 2007).

obviousness are different. Life does contend that if the Promega patents are not obvious (because *every* combination of loci is inventive, as Promega has argued) then the patents must also not be enabled (because patent fails to disclose how to make any combination work except those expressly disclosed, even as they cover countless more). Moreover, while Life moved for summary judgment of obviousness contingent on its enablement claim being rejected, its *opposition* to Promega's summary judgment never stated its obviousness position for *trial* was contingent on the enablement issue.

Second, the district court's assertion that Life's expert did not include an obviousness opinion is factually wrong. *See* A936, A940, A951-952 [Struhl Invalidity Report] ¶¶ 4, 13, 45 ("In my opinion, the Promega patents do not teach any improvement or advancement beyond what was already known by skilled artisans, and in fact teach far less than what was already known. . . . In the event that the Promega patents are actually deemed to teach and enable skilled artisans to multiplex sets of loci other than those listed in the claims, it would be my opinion that the claims would have been obvious in light of the prior art."). But even if he had not, there is no requirement that an expert opine on the legal question of obviousness. *See Soverain Software LLC v. Newegg Inc.*, 705 F.3d 1333 (Fed. Cir. 2013) (rejecting the argument that the absence of an expert opinion on obviousness dooms the defense).

Third, the district court placed much weight on its conclusion that there is no evidence that persons of ordinary skill in the art would know a priori that the claimed combinations of loci and particular primers could be successfully multiplexed. But this just treats unpredictability of particular results as sufficient to support inventiveness. As discussed above, that is wrong. It is not an invention to employ that obvious process to pursue an obvious goal.

In sum, the district court erred by granting summary judgment of obviousness. The claims are obvious and there is ample evidence of obviousness that at least creates factual disputes preventing the entry of summary judgment.

III. THE DISTRICT COURT ERRED BY INTERPRETING THE 2006 CROSS LICENSE TOO NARROWLY

Defendants are licensed to practice the Promega patents and the Tautz patent in the fields of "Forensics and Human Identity Applications," pursuant to the parties' 2006 Cross License. These fields include

See A816,

819 [2006 Cross License Agreement] §§ 1.6, 1.7, 2.1.2.

When the parties disputed whether part (a) of this license grant covered forensic research, education, and training, Promega took an unduly narrow view. For instance, with regard to forensic education, Promega successfully argued

below that forensic education in a forensic training program at a university could never be "in preparation for" legal proceedings because students in such training programs "are not running off to a legal proceeding." See A1660 [Promega's Memo In Support of Untimely Mot.]; see also A1656 ("Students do not go from the classroom to a legal proceeding."). Promega argued similarly that the license scope was narrow as to forensic research and training. The district court ultimately adopted Promega's narrow view, orally ruling that "it's just obvious" that part (a) of the 2006 license grant covers only forensic applications that are part of ongoing police investigations or are "connected" to "law enforcement agencies." See A1792 [2/6/2012 Trial Tr.] at 19:1-4 ("I think it's just obvious that research, educational and training is not covered unless it's done right there in the police department. But you're talking about genetic research that's done at universities that are not connected with any law enforcement agencies.").

A. THE PLAIN LANGUAGE OF THE LICENSE GRANT CONFIRMS DEFENDANTS' INTERPRETATION

The 2006 Cross License provides that Forensic and Human Identity Applications encompass DNA analysis for "use in, *or* in preparation for, legal proceedings." In other words, the 2006 Cross License contemplates two types of forensic uses: (1) those that are for "use in" legal proceedings, and (2) those that are simply "in preparation for" legal proceedings. Forensic applications of Life's STR kits associated with a live case or that are carried out by law enforcement

agencies are clearly for "use in" legal proceedings. However, in limiting the 2006 Cross License to just these applications, the Court ignored that forensics research, education, and training are prerequisites for and necessary parts of any use in a legal proceeding. This is so because forensics standards bodies (e.g., the FBI) mandate that the specific STR kits sold by any manufacturer (including Life) (1) be validated and tested and (2) be used in the training and education of forensic scientists who will ultimately use the kits in live criminal investigations. See A1593 [Declaration of A. Eisenberg] ¶ 3; see also Section III.B., below (discussing validation, testing, and training in more detail). It is difficult to see how forensic research, education, and training activities that, for example, are mandated in standards set forth by forensic bodies such as the FBI and Forensic Science Education Programs Accreditation Commission do not qualify as Forensic and Human Identity Applications that are part and parcel of "use" in legal proceedings.

In addition, the 2006 Cross License's use of the word "or" provides for a host of broader uses that are simply "in preparation for" legal proceedings. Even if forensics research, education, and training could properly be interpreted as not within the scope of the "use" of STR kits in legal proceedings, certainly they represent uses that are "in preparation for" legal proceedings. As discussed above, the mandates of the forensics standards bodies require forensic research, education, and training activities as prerequisites to presenting STR kit-based evidence in

legal proceedings. Indeed, the raison d'être of the entire field of forensics is to investigate and establish facts in criminal or civil courts, and it thus cannot be said that activities mandated by the bodies that govern this very field are not in "in preparation for" legal proceedings.

B. THE COURT'S INTERPRETATION PREVENTS DEFENDANTS FROM ENJOYING THE BENEFIT OF THE 2006 CROSS LICENSE

Excluding forensic research, education, and training activities from the scope of the 2006 Cross License is not just inconsistent with the plain language of the license grant, but is also an interpretation that would preclude Life from enjoying the full benefit of the bargain it struck in the 2006 Cross License.

Two principles of contract interpretation are central to a proper analysis. First, a "contract must be so interpreted as to give effect to the mutual intention of the parties as it existed at the time of contracting, so far as the same is ascertainable and lawful." Cal. Civ. Code. § 1636. Second, a "contract must receive such an interpretation as will make it lawful, operative, definite, reasonable, and capable of being carried into effect, if it can be done without violating the intention of the parties." *Id.* § 1643. As a general matter, "[t]he basic premise of contract law is to effectuate the expectations of the parties to the agreement, to give them the benefit of the bargain they struck when they entered into the agreement." *Nat'l Rural Telcoms. Coop. v. DIRECTV, Inc.*, 319 F. Supp. 2d 1040, 1048 (C.D. Cal. 2003); *see also Safeco Ins. Co. v. Robert S.*, 28 P.3d 889, 894 (Cal. 2001) ("When

reasonably practical, contracts are to be interpreted in a manner that makes them reasonable and capable of being carried into effect, and that is consistent with the parties' intent.").

In concluding that the 2006 Cross License excludes forensic training, research, and education unless it is part of an ongoing criminal investigation ("done right there in the police department") or "connected" with a "law enforcement agency," the district court ignored these principles. As noted above, forensics standards bodies (e.g., the FBI) mandate that the STR kits sold by any manufacturer (including Life) (1) be validated and tested and (2) be used in the training and education of forensic scientists who will ultimately use the kits in live criminal investigations. These activities constitute forensic research, education, and training, and they are gateway applications predicate to direct "use in" legal proceedings because if they are not carried out for every specific type of STR kit that defendants sell or plan to sell, forensic scientists cannot properly use the kits in legal proceedings. See A1593 [Declaration of A. Eisenberg] ¶ 3. Accordingly, Life must be able to supply STR kits for forensic training, research, and education or it will lose the benefit of the bargain it struck in the 2006 Cross License.

For instance, with regard to forensic research, the FBI QAS sets forth validation standards that apply to all forensic techniques of DNA analysis, including the analytical techniques embodied in the STR kits sold by Life.

Specifically, the QAS mandates that "[d]evelopmental validation shall precede the use of a novel methodology for forensic DNA analysis," a process that includes research activities requiring the use of STR kits, such as research into the genetic markers used, sensitivity studies, stability studies, population studies, etc. See A1616 [Quality Assurance Standards] ¶¶ 8.2, 8.2.1 All of this work is done outside a police lab and not necessarily by a law enforcement agency. Yet, it is used—indeed essential—in legal proceedings because it is the only basis for a forensic scientist to testify regarding the probability of a DNA profile match. See A1593 [Declaration of A. Eisenberg] ¶ 5. For instance, population studies to assess the frequency of genetic variation among populations are used in legal proceedings because they are required to establish the statistical significance of a profile match. *Id.* Likewise, research into the limitations and parameters of the test (e.g., DNA collection and extraction techniques used, detection parameters, sensitivity, etc.) are foundational for use in legal proceedings because they establish the reliability of a method. See A1594 ¶ 6.

The FBI QAS mandates additional "internal validation" procedures that involve accumulation of test data *within* a laboratory to confirm that the methods perform as expected in the laboratory. *See* A1594 [Declaration of A. Eisenberg] ¶ 7. Pursuant to the FBI QAS, every individual forensic laboratory is required to carry out its own studies to establish its own guidelines for interpretation of data.

Promega's expert agrees that "[e]ach crime lab must also validate these kits." A1663-64 [Declaration of J. Ballantyne] ¶ 8. These validation activities—which fall into the category of forensic research—are thus mandated before a forensic laboratory can utilize Life's STR kits in legal proceedings, and therefore are clearly "in preparation for" legal proceedings. *See* A1595 [Declaration of A. Eisenberg] ¶ 9. Simply put, while Promega argued before the district court that there was a "tenuous link between basic forensic research and legal proceedings," A1660, the exact opposite is true.

Forensic education presents a situation similar to forensic training. The Forensic Science Education Programs Accreditation Commission ("FEPAC") has issued Accreditation Standards for undergraduate and graduate programs in the forensic sciences. *See* A1630-1641 [Forensic Science Education Programs]. These standards mandate the use of laboratory-based instruction, including actual use of STR kits by students and instructors. *See* A1596 [Declaration of A. Eisenberg] ¶ 13; *see also* A1645-1646 [Ballantyne Depo. Tr.] at 14:21-15:9 (Promega's expert confirms that the forensics training curriculum at the University of Florida involves teaching using actual STR kits). The FBI has issued a set of Quality Assurance Standards for Forensic DNA Testing Laboratories ("QAS"). A1600-1627 [Quality Assurance Standards]. These standards have become the benchmark for assessing forensic DNA laboratories, and all forensic testing for use

in or in preparation for legal proceedings must comply with the FBI's QAS. *See* A1592-93, A1597 [Declaration of A. Eisenberg] ¶¶ 2, 16. Most important, the QAS mandates hands-on experience with the specific tools (including STR kits) that forensic analysts ultimately use in legal proceedings. *See* A1597-98 [Declaration of A. Eisenberg] ¶ 18; *see also* A1609 [Quality Assurance Standards] ¶ 5.1.2.2.3 ("All analyst/technician(s), regardless of previous experience, shall successfully complete a competency test(s) covering the routine DNA methodologies to be used prior to participating in independent casework analysis").

Thus, if forensic analysts cannot be trained and tested in the use of Life's STR kits pursuant to the FBI QAS, they cannot use them in casework, and of course they will stop ordering them from Life, which inexorably means Life would not enjoy the full benefit of the 2006 Cross License under its proper interpretation. See A1598-99 [Declaration of A. Eisenberg] ¶ 20. Notably, before the district court, Promega did not challenge that this sort of training was mandated by the FBI QAS, and did not disagree that forensic scientists would likely quickly stop ordering Life's STR kits if they cannot use them to carry out this mandated training. See generally A1647-1661 [Promega's Memo In Support of Untimely Mot.]. The same is true for those trained in undergraduate and graduate forensics programs. See A1595 [Declaration of A. Eisenberg] ¶ 11.

Before the district court, Promega argued that "[t]o say someone sitting in a classroom . . . is preparing for legal proceedings is an absurdity." A1660. In fact, even more than a law student is preparing for legal proceedings while "sitting in a classroom," a student in a forensic training program is preparing for a legal proceeding while in the classroom. As explained above, the forensics student or trainee is learning specifically how to use the very kits that will be used for legal proceedings, and *cannot* use those kits in legal proceedings if he or she has not been trained on them. Thus, if Life is precluded from making its kits available for these activities, it will be unable to enjoy fairly the benefit of its 2006 Cross License in the forensic market.

Simply put, the uses identified above are gateway applications of Life's STR kits necessary for them to be used in legal proceedings as contemplated by the parties. To conclude that Life cannot supply STR kits for these purposes would work a *de facto* obstacle to forensic scientists subsequently using Life's STR kits for legal proceedings. Quickly, Life's kits would fall out of favor among for scientists and the market for them would shrink, leaving defendants unable to enjoy the benefit of the bargain they struck in the 2006 Cross License. *See* A1598-99 [Declaration of A. Eisenberg] ¶ 20. The text of the license grant does not support such a self-contradictory reading of the license, and neither does common sense. Under California law, "[a] fair and reasonable interpretation is always

preferred rather than one leading to harsh and unreasonable results." *Bergin v. Van Der Steen*, 236 P.2d 613, 617 (Cal. App. 1951); *see also* Cal. Civ. Code. §§ 1636, 1643.

C. AT A MINIMUM OPEN CONTRACT INTERPRETATION QUESTIONS SHOULD HAVE BEEN SUBMITTED TO THE JURY

During pre-trial proceedings, the parties' submitted conflicting extrinsic evidence as to whether the 2006 Cross License agreement encompasses forensic education, research, and training. Defendants submitted an eight page expert witness declaration, the FBI QAS, the FEPAC Accreditation Standards, and deposition testimony from Promega's expert. *See generally* A1576-1589; A1591-1599. For its part, Promega submitted its own competing expert witness declaration, a declaration from its Chief Technology Officer and Vice-President, deposition testimony from Life's head of product management, deposition testimony from a Life sales representative, and four scientific publications in the area of forensics research. *See generally* A1647-1661; *see* A1690-1691 [2/3/2012 Hearing Tr.] at 22:17-23:1.

At a minimum, in view of the substantial volume of conflicting extrinsic evidence submitted, the district court should have submitted the contract interpretation question to the jury. Indeed, under California law, "when there is a material conflict in extrinsic evidence supporting competing interpretations of ambiguous contract language the court may not use the evidence to interpret the

for evaluation of its credibility." *Cachil Dehe Band of Wintun Indians*, 618 F.3d at 1077; *see also City of Hope v. Genentech Inc.*, 181 P.3d 142, 156 (Cal. 2008) ("But when, as here, ascertaining the intent of the parties at the time the contract was

contract as a matter of law, but must instead render the evidence to the fact finder

executed depends on the credibility of extrinsic evidence, that credibility

determination and the interpretation of the contract are questions of fact that may

properly be resolved by the jury."); City of Hope, 181 P.3d at 156 ("Juries are not

prohibited from interpreting contracts.").

CONCLUSION

For all the above reasons, (1) the Promega patents should be found not enabled, (2) the Promega patents should be found obvious, and (3) the 2006 Cross License should be interpreted to cover forensic education, research, and training.

Dated: July 12, 2013 Respectfully submitted,

/s/ Edward R. Reines

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ADDENDUM

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1	Court's Opinion and Order dated November 29, 2011	A1-A33
2	Court's Opinion and Order dated February 1, 2012	A34-A51
3	Court's Opinion and Order dated February 3, 2012	A52-A62
4	Excerpt of Trial Transcript dated February 6, 2012	A63-A69
5	Amended Judgment dated September 14, 2012	A70-A71
6	U.S. Patent No. 5,843,660	A205-A260
7	U.S. Patent No. 6,221,598	A261-A292
8	U.S. Patent No. 6,479,235	A293-A340
9	U.S. Patent No. 7,008,771	A341-A392
10	U.S. Patent No. RE37,984	A393-A408

TAB 1

IN THE UNITED STATES DISTRICT COURT FOR THE WESTERN DISTRICT OF WISCONSIN

PROMEGA CORPORATION,

OPINION AND ORDER

Plaintiff,

and 10-cv-281-bbc

MAX-PLANCK-GESELLSCHAFT zur FORDERUNG der WISSENSCHAFTEN E.V.,

Involuntary Plaintiff,

v.

LIFE TECHNOLOGIES CORPORATION, INVITROGEN IP HOLDINGS, INC. and APPLIED BIOSYSTEMS, LLC,

Defendants.

Plaintiff Promega Corporation is suing defendants Life Technologies Corporation, Applied Biosystems, LLC and Invitrogen IP Holdings, Inc. for infringement of U.S. Patents Nos. 5,843,660, 6,221,598, 6,479,235, 7,008,771 and Re 37,984. (Both sides treat the three defendants as one entity for the purpose of the motions for summary judgment, so I will do the same.) Plaintiff owns the first four patents and is the exclusive licensee of

involuntary plaintiff Max Planck with respect to the fifth. The patents relate to "multiplex amplification of short tandem repeat loci," which are regions on a DNA strand that contain repeating nucleotide sequences. Because the number of repeats of particular sequences can vary greatly from person to person, these differences can be used to compare different DNA samples for possible matches. To facilitate the process, the loci are copied, or "amplified." "Multiplex" amplification means that multiple loci are copied simultaneously to make the process more efficient.

The asserted patents include both apparatus and method claims. Plaintiff contends that kits made and sold by defendants directly infringe the apparatus claims and that defendants induce infringement of the method claims. The asserted apparatus claims are claims 18-19 and 21-23 of the '235 patent, claims 10, 23-24, 27 and 33 of the '598 patent; claims 25 and 27-31 of the '660 patent, claim 5 of the '771 patent and claim 42 of the '984 patent. The asserted method claims are claims 1-4, 6-13 and 15-17 of the '235 patent, claims 1-2, 4-5, 7-9, 12, 15, 19, 21-22, 28 and 31-32 of the '598 patent; claims 2-5, 16-17, 19-21 and 23-24 of the '660 patent and claims 15-16, 18, 23, 25, 27-28 and 41 of the '984 patent.

Plaintiff has filed a motion for summary judgment with respect to infringement of all five patents as well as on defendants' invalidity defenses and counterclaims for anticipation, lack of enablement and obviousness. Defendants have filed a motion for partial summary

judgment for noninfringement, lack of enablement and obviousness with respect to all of the patents except the '984 patent.

I am granting defendants' motion with respect to noninfringement of claims 25 and 27-31 of the '660 patent because I conclude that those claims are limited to products that use no loci other than those listed in the claims and the parties agree that none of the accused products are limited to just those loci. Because the remaining asserted claims are open-ended (they do not exclude unrecited loci) and the parties identify no other potential differences between the accused products, I am granting plaintiff's motion for summary judgment with respect to direct infringement of all other claims that disclose a kit. I disagree with defendants that their sale of the kits is covered by a license agreement with plaintiff and that plaintiff lacks standing to sue under the '984 patent.

With respect to the method claims, plaintiff is not seeking summary judgment for direct infringement, only for inducement under 35 U.S.C. § 271(b). I am denying plaintiff's motion for summary judgment with respect to inducement and willfulness because plaintiff failed to develop arguments on these issues. Because defendants' motion for summary judgment did not include these issues, they will have to proceed to trial.

With respect to invalidity, I conclude that plaintiff is entitled to summary judgment on defendants' affirmative defenses and counterclaims of anticipation, obviousness and lack of enablement. The enablement defense is contingent on an incorrect view that the

patentees were required to enable unrecited elements and defendants have failed to adduce any evidence that at the time the patent applications were filed, it would have been obvious to a person of ordinary skill in the art that the combinations of loci disclosed in the asserted patents could coamplify successfully.

Defendants do not contend in their summary judgment briefs that any of the claims in the asserted patents are anticipated, but they say that the court should not rule on this issue because they never raised it. I disagree. Although it is true that defendants did not include an opinion on anticipation in their expert report, in their answer they included an affirmative defense and a counterclaim that "the '660, '598, '235, and '771 patents are invalid for failure to comply with one or more of the requirements of the United States patent laws, including at least 35 U.S.C. sections 102, 103 and/or 112." Ans., dkt. #150, at 35. Anticipation is one of the defenses under 35 U.S.C. § 102. Defendants did not explicitly identify anticipation as a defense or a counterclaim, but they did not identify any other particular invalidity defenses either. Thus, if defendants properly raised any invalidity defenses in their answer and counterclaim, anticipation was among them. Accordingly, I conclude that there is an actual controversy regarding that issue and that plaintiff is entitled to summary judgment because defendants failed to show that a genuine issue of material fact exists.

Two other motions are before the court: (1) plaintiff's motion to "strike" defendants'

brief in support of their motion for partial summary judgment, or, in the alternative, to disregard any facts not included in defendants' proposed findings of fact, dkt. #262; and (2) plaintiff's motion for leave to file a reply brief in support of the motion to strike. Dkt. #293. With respect to the motion to strike, I will grant plaintiff's alternative request because the court's procedures are clear that "[a]ll facts necessary to sustain a party's position on a motion for summary judgment must be explicitly proposed as findings of fact." Helpful Tips for Filing a Summary Judgment Motion, Tip #1, dkt. #69, at 11. See also Procedure to Be Followed on Motions for Summary Judgment, I.B.4, dkt, #69, at 14 ("The court will not consider facts contained only in a brief."). I have not considered facts submitted by either side unless they were included in its proposed findings of fact. Plaintiff's motion to file a reply brief will be denied as unnecessary.

BACKGROUND

Certain locations or "loci" on chromosomes vary from individual to individual. These are called polymorphic loci and are useful as identifiers. However, no one locus will positively identify an individual to a statistically significant degree because no one locus is unique to each individual within any given population.

Short tandem repeats (STRs) are loci found within genomic DNA that have a number of short repetitive nucleotide sequences. The DNA sequences at a particular STR locus

within a given population will exhibit a variable number of these repeat sequences. It is this variation in the number of repeats at a particular locus that is responsible for the polymorphisms that permit scientists to genetically distinguish one individual from another.

Polymerase chain reaction is one method of amplifying loci. There are several steps in the process. First, the two strands of genomic DNA are heated and then separated to form "single stranded" DNA. Second, a pair of "primers" is introduced and allowed to pair with the single stranded DNA. This pairing occurs in accordance with the nucleotide pairing rules, that is, at a point on the single stranded DNA where the primer sequence is complementary to the genomic nucleotide sequence.

Amplifying the alleles present at a single locus is commonly referred to as a "monoplex" reaction. Amplifying multiple STR loci simultaneously is a "multiplex" reaction. To minimize labor, materials and analysis time, it is desirable to analyze multiple loci and samples simultaneously. One approach for reaching this goal involves amplification of multiple loci simultaneously in a single reaction.

The amplified alleles from one DNA sample can be compared to the amplified alleles of a second DNA sample by, for example, running the two samples side-by-side on the gel. One can then determine whether the two samples came from the same individual. Additionally, a "size marker" or "allelic ladder" is often run concurrently with the sample in another lane of the gel. By comparing the alleles amplified in the DNA sample to the allelic

ladder one can determine precisely which alleles appear in the DNA sample.

Defendants manufacture, offer for sale and sell AmpFISTR Amplification Kits. These kits provide components for carrying out simultaneous amplification of multiple short tandem repeat loci from one or more DNA samples. The kits are used for chimerism in the context of bone marrow transplant monitoring, cell line authentication, genotyping hydatidiform moles, cancer analysis, determinations of fetal sex and anthropological research, among other things.

Chimerism occurs following bone marrow transplantation when the recipient produces her own blood cells as well as donor blood cells. The kits are used to compare the amount of amplified STR alleles from the donor and host and then to determine the proportion of blood cells contributed by each source. Repetitive testing over time indicates whether the proportion of blood cells from the donor and host is changing, which has treatment and prognostic value.

In genotyping hydatidiform moles, kits are used to classify moles in a woman's uterus during pregnancy to assess whether the woman is at risk for particular diseases. In cell line authentication, kits are used to determine whether new cell lines are unique. In cancer analysis, the kits are used to analyze genetic instability in cancers by detecting allelic imbalance.

OPINION

A. Claim Construction

The parties' arguments on questions of infringement and invalidity rely in part on their understanding of the phrase "a set of . . . loci," which appears in all of the asserted claims in the '235, '298, '660, and '771 patents. In particular, each claim includes the phrase "a set of . . . loci" followed by a list of particular loci. For example, claim 16 of the '660 patent discloses:

A method of simultaneously determining the alleles present in three short tandem repeat loci from one or more DNA samples, comprising:

- (a) obtaining at least one DNA sample to be analyzed,
- (b) selecting a set of three short tandem repeat loci of the DNA sample to be analyzed which can be amplified together, wherein the set of three loci is selected from the group of sets of loci consisting of:

D3S1539, D19S253, D13S317; D10S1239, D9S930, D20S481; D10S1239, D4S2368, D20S481; D10S1239, D9S930, D4S2368; D16S539, D7S820, D13S317; and D10S1239, D9S930, D13S317.

- (c) co-amplifying the three loci in the set in a multiplex amplification reaction, wherein the product of the reaction is a mixture of amplified alleles from each of the co-amplified loci in the set; and
- (d) evaluating the amplified alleles in the mixture to determine the alleles present at each of the loci analyzed in the set within the DNA sample.

The question of claim construction presented by the parties is whether the set may include loci in addition to those that are listed in the claim, that is, whether the set is open or closed. Plaintiff says all of the asserted claims are open-ended; defendants say they are all closed.

The parties raised this issue in their claim construction briefs, but I declined to resolve it because both sides supported their arguments with text of particular claims without accounting for the textual differences among the claims. Accordingly, I directed the parties to reassert their arguments at summary judgment if they believed a construction was needed to resolve a dispute of infringement or invalidity. "In the meantime, the parties should consider how they wish to frame their arguments. If they believe that 'a set of . . . loci' has an identical meaning everywhere it appears in every asserted claim in every asserted patent, then they should be prepared to explain why textual differences in the claims may be disregarded. They should not use the language of a particular claim to support a construction they wish to be applied across the board." Dkt. #190, at 4.

Defendants largely disregarded these instructions in their summary judgment materials. They advance arguments from the prosecution history with the assumption that a statement from the history of one patent applies equally to another and they cherry pick language from particular claims while ignoring other claims that have different texts.

I will consider defendants' arguments about the prosecution history first and I will assume that any statement in the prosecution history applies to all of the asserted patents.

Defendants argue that the applicants disclaimed the inclusion of any loci in the reaction not expressly listed in the claims. In support, defendants cite various statements from the applicants that the prior art did not include "these combinations" of loci and a statement from the examiner of the '598 patent that the prior art "does not teach the specific combinations provided in the claims." Dfts.' Br., dkt. #245, at 12-13. (Defendants did not include proposed findings of fact about these aspects of the prosecution history, but I will consider them because doing so will not make any difference to the outcome of the motion.)

If the applicants had been distinguishing prior art that included one of the listed sets of loci *and* one or more additional loci, defendants would have a stronger argument of disclaimer. Defendants' argument fails because the applicants were distinguishing prior art that was *missing* some of the loci in the listed combinations. For example, the applicants noted that Oldroyd included two of the loci listed in claim 1 of '660 patent, but none of the other loci. Dkt. #240-12. Thus, a statement that "these combinations" were not in the prior art does not disclaim an invention that includes those combinations and additional loci.

The cases defendants cite provide no support for their argument. In <u>Seachange International, Inc. v. C-COR, Inc.</u>, 413 F.3d 1361, 1369 (Fed. Cir. 2005), the question was whether the applicants had defined the term "network for data communications" to mean "point-to-point networks" during the prosecution history. In concluding that they had, the

court of appeals relied on statements in which the applicants overcame an examiner's objection by explaining that the prior art did not include a point-to-point network. In Elkay Manufacturing Co. v. Ebco Manufacturing Co., 192 F.3d 973, 977 (Fed. Cir. 1999), the question was whether the term "an upstanding feed tube" meant one tube or could mean more than one. The court limited the term to one tube because, during the prosecution history, the applicants had distinguished prior art on the ground that it used multiple tubes.

Neither of these cases raised the question whether the claimed invention is limited to recited items. Both involved applicants who needed to narrowly define their invention during prosecution in order to overcome an anticipation defense. Because the applicants in this case did not define their invention to exclude additional loci, <u>SeaChange</u> and <u>Elkay</u> are not on point.

Defendants' other "universal" argument is similar. They rely on Smith v. Snow, 294 U.S. 1, 14 (1935), Phillips v. AWH Corp., 415 F.3d 1303, 1321 (Fed. Cir. 2005), Acumed, LLC v. Stryker Corp., 483 F.3d 800, 815 (Fed. Cir. 2007), and In re Gray, 53 F.2d 520, 522 (CCPA 1931), for the proposition that claims should not be construed to cover more than what was actually invented. Because the applicants did not invent any combinations of loci other than those listed in the claims, defendants say it would violate this principle to allow the claims to cover additional loci.

Again, none of the cited cases raise the question whether a claim must be limited to

recited elements. It is well-established that claims are not so limited; that is the whole point of using terms such as "comprising" or "including." Crystal Semiconductor Corp. v. TriTech Microelectronics International, Inc., 246 F.3d 1336, 1348 (Fed Cir. 2001) ("[T]he transition 'comprising' creates a presumption that the recited elements are only a part of the device, that the claim does not exclude additional, unrecited elements."); AFG Industries, Inc. v. Cardinal IG Co., Inc., 239 F.3d 1239, 1244 (Fed. Cir. 2001) ("When a claim uses an 'open' transition phrase, its scope may cover devices that employ additional, unrecited elements."); Stiftung v. Renishaw PLC, 945 F.2d 1173, 1178 (Fed. Cir. 1991) (a claim that "uses the term 'comprising,' is an 'open' claim which will read on devices which add additional elements"). If I were to accept defendants' argument, it would mean that a defendant could avoid infringement simply by adding more elements to a device or method. That is not the law, even when the additional elements are an improvement to the claimed invention. Free Motion Fitness, Inc. v. Cybex International, Inc., 423 F.3d 1343, 1347 (Fed. Cir. 2005) ("The addition of unclaimed elements does not typically defeat infringement when a patent uses an open transitional phrase such as 'comprising.'"); Lighting World, Inc. v. Birchwood Lighting, Inc., 382 F.3d 1354, 1365 (Fed. Cir. 2004) ("Making improvements on a patented invention by adding features to a claimed device beyond those recited in the patent does not avoid infringement."); A.B. Dick Co. v. Burroughs Corp., 713 F.2d 700, 703 (Fed. Cir. 1983) ("It is fundamental that one cannot

avoid infringement merely by adding elements if each element recited in the claims is found in the accused device."). See also Gillette Co. v. Energizer Holdings, Inc., 405 F.3d 1367, 1374 (Fed. Cir. 2005) (claim disclosing razor with three blades could read on razor with four blades); Genentech, Inc. v. Chiron Corp., 112 F.3d 495, 499 (Fed. Cir. 1997) ("[T]he district court improperly limited the transitional phrase 'comprising,' which allows additional elements to be present as long as the named elements are present, to exclude additional DNA between the alpha-factor processing sequences and the human IGF-I sequence.").

Turning to the language of the asserted claims, I will begin with the '660 patent because I construed some of those claims in a previous case. <u>Promega Corporation v. Applera Corporation</u>, No. 01-C-244-C (W.D. Wis. June 10, 2002), dkt. #64. The question in case no. 01-C-244-C was the same as in this case, whether "a set of . . . loci" in the asserted claims was opened or closed. In this case, plaintiff is asserting claims 2-5, 16-17, 19-21, 23-25 and 27-31; in case no. 01-C-244-C, plaintiff was asserting claims 1-5 and 16. Although claim 1 is not asserted in this case, it is relevant because claims 2-5 depend from it. Claim 1 discloses:

A method of simultaneously determining the alleles present in at least four short tandem repeat loci from one or more DNA samples, comprising:

- (a) obtaining at least one DNA sample to be analyzed,
- (b) selecting a set of at least four short tandem repeat loci of the DNA sample to be analyzed which can be amplified together, wherein the at least four loci

in the set are selected from the group of loci consisting of:

D3S1539, D4S2368, D5S818, D7S820, D9S930, D10S1239, D13S317, D14S118, D14S548, D14S562, D16S490, D16S539, D16S753, D17S1298, D17S1299, D19S253, D20S481, D22S683, HUMCSF1PO, HUMTPOX, HUMTH01, HUMF13A01, HUMBFXIII, HUMLIPOL, HUMvWFA31;

- (c) co-amplifying the loci in the set in a multiplex amplification reaction, wherein the product of the reaction is a mixture of amplified alleles from each of the co-amplified loci in the set; and
- (d) evaluating the amplified alleles in the mixture to determine the alleles present at each of the loci analyzed in the set within the DNA sample.

A review of the 2002 opinion reveals that there were *two* disputes about the scope of the claims, both of which seem to be relevant to this case. The first was the one focused on by the parties in this case, that is, whether the list of loci in step (b) is exclusive or may include other unnamed loci. The second was whether step (c) may include loci not listed in step (b), regardless whether the list in step (b) is closed. Both sides raise arguments about both issues, though neither acknowledges that the issues are distinct. In any event, the parties seem to agree that the accused products infringe the claims of the '660 patent if plaintiff prevails on either issue.

In case no. 01-C-244-C, I agreed initially with the defendants that lists of loci identified in claims 1-5 and 16 were closed and that the loci in step (c) were limited to the list in step (b). Promega Corporation v. Applera Corporation, No. 01-C-244-C (W.D. Wis. Jan. 3, 2002), dkt. #40. However, upon reconsideration, I adopted the following

construction: "Claims 1 through 5 and 16 of the '660 Patent require the presence of at least one of the sets identified in the Markush groups stated in limitation (b) of those claims but do not exclude the presence of other STR loci in the multiplex reaction required by limitation (c) of those claims." Dkt. #64, at 10. In reaching that conclusion, I discussed several factors.

First, I concluded that it was important not to conflate the loci in "the set" in step (b) with the loci in the "reaction" in step (c). That is, even if "the set" in step (b) was limited to the recited loci, it did not follow that the loci in the "multiplex amplification reaction" in step (c) was limited to those listed in step (b). I concluded that the language of step (c) did not exclude the presence of other loci. (Plaintiff buttresses that conclusion in this case by pointing out that step (c) discloses a "mixture," which generally permits ingredients not listed in the claim. Mars, Inc. v. H.J. Heinz Co., 377 F.3d 1369 (Fed. Cir. 2004).)

Second, I cited the rule that "[o]ne who does not infringe an independent claim cannot infringe a claim dependent on (and thus containing all the limitations of) that claim." Wahpeton Canvas Co., Inc. v. Frontier, Inc., 870 F.2d 1546, 1552 (Fed. Cir. 1989). Under the defendants' view, this rule would be broken because it would be possible for an accused product to infringe a dependent claim without infringing the independent claim. For example, claim 3 contains the following set of loci: "D16S539, D7S820, D13S317, D5S818, HUMFI3A01, HUMFESFPS." Although the first five of these loci are listed in claim 1,

HUMFESFPS is not. Thus, if the set of loci in claim 1 is closed, a product that included the six loci in claim 3 could infringe claim 3, but not claim 1.

Third, I rejected the defendants' argument that the patentees disclaimed an open set when they amended the phrase "at least four of the loci in the set" to "the at least four loci in the set." Although I acknowledged the possibility that inclusion of "the" could be read "to require that all the loci in a set, whether four or more, be selected from the Markush group in step (b)," I also found credible plaintiff's argument that "the amendment was not substantive, but was made instead to conform the claim to standard patent claim drafting procedure, which requires that an element of a claim be preceded by a definite article, such as 'the,' each time it is referred to after its initial appearance in a claim." Dkt. #64, at 8-9. Because neither the patentees nor the examiner made a clear statement regarding the amendment's significance, I declined to narrow the scope of the claim.

Finally, I cited a statement by the patentees when they deleted the HUMFESFPS loci from the list in claim 1: "the amendments to claim 1 do not change the fact that the claimed method encompasses the coamplification and evaluation of sets of short tandem repeat loci which include the deleted locus, provided at least four of the loci in the set . . . are selected from the remaining group of loci listed in claim 1." Because there was no clear evidence that the patentees ever disavowed this broad interpretation or that the examiner disagreed with it, the statement supported plaintiff's view that the set was open.

As I noted in the claim construction order in this case, the law suggests that I am not bound by the conclusion in the 2002 opinion because the case settled before judgment. Talmage v. Harris, 486 F.3d 968, 974 (7th Cir. 2007) ("Normally, when a case is resolved by settlement or stipulation, courts will find that the 'valid final judgment' requirement of issue preclusion has not been satisfied."). However, defendants do not directly address the 2002 opinion or criticize its reasoning. Although they raise arguments that would conflict with the earlier case, those arguments are undeveloped and unpersuasive. Accordingly, I decline to depart from my previous conclusion.

This resolves the claim construction dispute with respect to claims 2-5 and 16 of the '660 patent. Because asserted claims 17, 19-21 and 23-24 all depend from claim 16 and do not include any additional "set of . . . loci" limitations, I need not consider those claims separately.

Claims 1-2, 4-5 and 7-9 of the '598 patent have a structure similar to that of claims 2-5 and 16 of the '660 patent. Because defendants do not point to any more restrictive language in claims 1-2, 4-5 and 7-9 of the '598 patent, I conclude that those claims may include unrecited loci as well.

Asserted claim 25 in the '660 patent is another matter. That claim discloses:

A kit for simultaneously analyzing short tandem repeat sequences in at least three loci, comprising a container which has oligonucleotide primers for co-amplifying a set of at least three short tandem repeat loci, wherein the set of loci are selected from the sets of loci consisting of:

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D3S1539, D19S253, D13S317;
D10S1239, D9S930, D20S481;
D10S1239, D4S2368, D20S481;
D10S1239, D9S930, D4S2368;
D16S539, D7S820, D13S317;
D10S1239, D9S930, D13S317;
D3S1539, D7S820, D13S317, D5S818;
D17S1298, D7S820, D13S317, D5S818;
D20S481, D7S820, D13S317, D5S818;
D9S930, D7S820, D13S317, D5S818;
D10S1239, D7S820, D13S317, D5S818;
D14S118, D7S820, D13S317, D5S818;
D14S562, D7S820, D13S317, D5S818;
D14S548, D7S820, D13S317, D5S818;
D16S490, D7S820, D13S317, D5S818;
D17S1299, D7S820, D13S317, D5S818;
D16S539, D7S820, D13S317, D5S818;
D22S683, D7S820, D13S317, D5S818;
D16S753, D7S820, D13S317, D5S818;
D3S1539, D19S253, D13S317, D20S481;
D3S1539, D19S253, D4S2368, D20S481;
D10S1239, D9S930, D4S2368, D20S481;
D16S539, D7S820, D13S317, HUMvWFA31;
D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, HUMTPOX;
D16S539, D7S820, D13S317, D5S818, HUMF13A01, HUMFESFPS;
D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, HUMTPOX,
HUMTH01;
D16S539, D7S820, D13S317, D5S818, HUMF13A01, HUMFESFPS,
HUMBFXIII:
D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, HUMTPOX,
HUMTH01, HUMvWFA31; and
D16S539, D7S820, D13S317, D5S818, HUMF13A01, HUMFESFPS,
HUMBFXIII, HUMLIPOL.
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Both sides recognize that the phrase "consisting of" signals a closed list. "In simple

terms, a drafter uses the phrase 'consisting of' to mean 'I claim what follows and nothing else.'" Vehicular Technologies Corp. v. Titan Wheel Intern., Inc., 212 F.3d 1377, 1383 (Fed. Cir. 2000). Extending that logic to this claim would mean that the set must include loci from the list and no other loci. Unlike claims 2-5 and 16, claim 25 does not include a counterpart to step (c) that would allow unrecited loci to be included in a mixture. In addition, no claims depend from claim 25 that recite loci not included in claim 25.

Plaintiff asks the court not to construe claim 25 as closed because the claim includes the term "comprising," which it says supports a construction that additional, unrecited loci may be included. Although plaintiff is correct that the term "comprising" is open-ended, as defendants point out, the term "'[c]omprising' is not a weasel word with which to abrogate claim limitations." Spectrum International Inc. v. Sterilite Corp., 164 F.3d 1372, 1380 (Fed. Cir. 1998). The context of the term is important. In claim 25, "'[c]omprising' appears at the beginning of the claim . . . The presumption raised by the term 'comprising' does not reach into each of the [elements] to render every word and phrase therein open-ended." Dippin' Dots, Inc. v. Mosey. 476 F.3d 1337, 1343 (Fed. Cir. 2007). In other words, the term "comprising" in claim 25 suggests that the kit may include elements other than "a container which has oligonucleotide primers for co-amplifying a set of at least three short tandem repeat loci," but it does not suggest that the set may include loci outside the list.

The importance of context is shown by comparison to asserted claim 10 of the '598

patent:

A kit for simultaneously analyzing short tandem repeat sequences in at least three loci, comprising:

a single container containing oligonucleotide primers for each locus in a set of at least three short tandem repeat loci, wherein the at least three short tandem repeat loci in the set comprises at least three loci selected from the group consisting of: [a listing of 20 sets of three loci].

In this claim, the applicants wrote that the set "comprises at least three loci selected from the" recited group, making it clear that the set may include other loci outside the group. Claim 25 of the '660 patent is missing similar language.

Alternatively, plaintiff relies on the phrase "at least three loci" in claim 25: "the fact that the sets themselves (from which to choose) are bigger than three loci makes it expressly clear additional loci can be selected." Plt.'s Br., dkt. #228, at 11. This argument makes no sense. If the listed sets were limited to two or three loci, then the phrase "at least three loci" might support an argument that additional loci must be present as well. However, because some of the listed sets have three loci and some have more than three, there is no reason to interpret "at least three loci" as anything other than an acknowledgment that some of the listed sets have more than three loci in them.

Accordingly, I conclude that claim 25 of the '660 patent is limited to the listed loci.

Because asserted claims 27-31 depend from claim 25, this conclusion extends to those claims

as well.

The language of the remaining asserted independent claims makes it clear that they are not limited to the recited loci because they all use the word "comprising" when listing the loci. '598 pat., claim 12 ("selecting a set of short tandem repeat loci of the DNA sample to be analyzed which can be co-amplified, comprising . . ."); <u>id.</u> at claim 23 ("a set of short tandem repeat loci which can be co-amplified, comprising . . ."); <u>id.</u> at claim 28 ("a set of short tandem repeat loci of the DNA sample to be analyzed which can be co-amplified, comprising . . . "); <u>id.</u> at claim 33 ("a set of short tandem repeat loci which can be co-amplified, comprising . . . "); <u>id.</u> at claim 33 ("a set of short tandem repeat loci which can be co-amplified, comprising . . . "); <u>id.</u> at claim 13 ("selecting a set of loci of the DNA sample, comprising . . . "); <u>id.</u> at claim 18 ("the loci comprise . . . "); '771 pat., claim 5 ("a set of loci from one or more DNA samples, comprising . . ."). The remaining asserted claims of these four patents are dependent claims that do not include more limiting language that is relevant to this issue. Accordingly, I conclude that all of the asserted claims allow for unrecited loci, with the exception of claims 25 and 27-31 of the '660 patent.

B. <u>Infringement</u>

Plaintiff contends that summary judgment is appropriate for direct infringement with respect to those asserted claims that disclose a kit and inducement of infringement with

respect to the method claims. Defendants do not deny in their briefs that the accused products include all of the elements of the '984 patent. With respect to the '660, '598, '235 and '771 patents, the only element defendants say is missing is "a set of . . . loci" on the ground that the accused products include loci not recited in the claims. In the previous section, I agreed with this argument with respect to claims 25 and 27-31 of the '660 patent, but I disagreed with respect to every other asserted claim. Accordingly, I will grant defendants' motion for summary judgment with respect claims 25 and 27-31 of the '660 patent, but I cannot grant defendants' motion on this ground with respect to the other asserted claims.

Defendants raise two more grounds for granting summary judgment with respect to direct infringement of the other asserted claims. First, defendants argue that any allegedly infringing acts under the '235, '598, '660 and '771 patents fall within the scope of a 1996 licensing agreement. Second, defendants argue that plaintiff does not have the right to sue under the '984 patent.

Finally, with respect to inducement, the question is whether plaintiff has proven inducement by defendants as a matter of law. Defendants have not moved for summary judgment on the question of inducement.

1. Direct infringement of the '235, '598, '660 and '771 patents: scope of 2006 cross license

The parties dispute whether several kinds of applications performed by the accused products sold by defendants fall within the scope of the license agreement: chimerism in the context of bone marrow transplant monitoring, cell line authentication, classifying molar specimens and determinations of fetal sex. The license extends to "any analysis, based on the measurement of the length of polynucleotide sequence containing a tandem repeat, of human genetic material for (a) use in, or preparation for, legal proceedings, or (b) analysis of biological specimens for the identification of individuals." Defendants argue that their kits fall within the scope of the license because they perform an "analysis of biological specimens for the identification of individuals."

Neither side cites much case law in favor of its position or even conducts a choice of law analysis. However, it is unnecessary to ask for supplemental briefing because it is clear from the plain language of the license and the undisputed facts that the kits in dispute do not perform an analysis "for the identification of individuals."

It is undisputed that the identity of the individual is either already known or irrelevant to the applications at issue. Plt.'s PFOF ¶ 135, dkt. #246; Dfts.' Resp. to Plt.'s PFOF ¶ 135, dkt. #257; Plt.'s PFOF ¶ 147, dkt. #246; Dfts.' Resp. to Plt.'s PFOF ¶ 147, dkt. #257; Plt.'s PFOF ¶ 150, dkt. #246; Dfts.' Resp. to Plt.'s PFOF ¶ 150, dkt. #257; Plt.'s PFOF ¶ 152, dkt. #246; Dfts.' Resp. to Plt.'s PFOF ¶ 152, dkt. #257. (Defendants dispute these proposed findings of fact on the ground that the applications involve a "human

identity application," but they do not dispute the fact that the identity of the individual is already known or irrelevant in each of them.) In particular, chimerism involves determining the relative *amount* present of two different types of DNA, Plt.'s PFOF ¶ 135; classifying molar specimens involves determining whether a mole is present and what type it is; Plt.'s PFOF ¶ 147; cell line authentication involves a determination whether two cell lines are unique, Plt.'s PFOF ¶ 149. Determination of fetal sex is self-explanatory.

Defendants do not dispute plaintiff's description of these applications, but they rely on the opinion of their expert for the proposition that the applications "determine the identity, or DNA fingerprint or genetic profile of a known individual." Booker Rpt., dkt. #291-1 at ¶17. That is not helpful. The expert's opinion suggests that the applications may be used for the identification of particular genetic characteristics, but it does not suggest that they are used "for the identification of *individuals*." Defendants do not provide any reason to give the word "individuals" anything other than its ordinary meaning.

To the extent the parties' subjective intent is relevant, the available evidence does not support defendants' view. For example, defendants' corporate representative, Daniel Hall, testified that defendants did not have a license from plaintiff for bone marrow transplant applications, which is evidence that defendants themselves do not believe that the license covers applications in which the identity of the donor is already known. Hall Dep., dkt. #233-48, at 53-54. Defendants do not even attempt to reconcile the representative's

position with their position in their summary judgment briefs that bone marrow transplant applications fall within the scope of the license. Accordingly, I am granting plaintiff's motion for summary judgment with respect to direct infringement of the asserted apparatus claims in the '235, '598, '660 and '771 patents, with the exception of claim 25 in the '660 patent and the claims that depend from claim 25.

2. Direct infringement of the '984 patent: scope of 1996 license

Defendants' argument on the '984 patent seems to be that plaintiff lacks standing to sue for infringement, though defendants do not say this explicitly. Rather, they say that plaintiff's rights under the '984 patent derive from a 1996 license that does not include the "research market" and that all of defendants' sales fall within that exception.

It is undisputed that plaintiff's rights under the '984 patent come from the 1996 license. Under that agreement, plaintiff has "an exclusive, worldwide license . . . for the HUMAN GENETIC IDENTITY and the HUMAN CLINICAL MARKET" except for "HUMAN LINKAGE ANALYSIS in the RESEARCH GENETICS FIELD OF USE." Dkt. #1-6. Defendants are simply wrong when they say that the agreement excludes the "research market" generally and they identify no reason to believe that any of their sales fall outside the human genetic identity market or the human clinical market.

Alternatively, defendants say that summary judgment is "premature" because the

parties are "in the midst of arbitration proceedings" that "could result in [plaintiff] losing all rights to the ['984] patent." Dfts.' Br., dkt. #253, at 31. Defendants provide no details and they cite no authority to support this view. I decline to stay a ruling on summary judgment because of an arbitration proceeding that may or may not affect plaintiff's rights in this case at some point in the future.

Although plaintiff asserted in its opening brief that the accused products meet all of the elements of the asserted claims in the '984 patent, defendants did not challenge this assertion in their opposition brief regarding noninfringement of this patent. Accordingly, I conclude that plaintiff is entitled to summary judgment with respect to infringement of the '984 patent.

3. Inducement of the method claims

Plaintiff said little about its claim that defendants may be held liable for inducing infringement under 35 U.S.C. § 271(b). It simply summarizes the standard and then lists a number of alleged actions by defendants. It did not develop any argument in support of a view that any of these actions constitute inducement or specify which actions induce infringement of which claims. Accordingly, plaintiff has not met its burden to show that it is entitled to judgment as a matter of law on its claims under § 271(b). Because defendants did not move for summary judgment on this issue, it will proceed to trial.

C. Enablement as to the '235, '598, '660 and '771 patents

Defendants' lack of enablement argument is the flip side of its noninfringement argument, that is, if the asserted claims are not limited to the recited loci, defendants say, they are invalid because the specification does not explain how to practice any methods or kits that use loci other those recited in the claims and undue experimentation would be required to determine what other loci could be added.

Defendants' argument is not persuasive. They cite the standard that "[t]o meet the enablement requirement, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without undue experimentation." Martek Biosciences Corp. v. Nutrinova, Inc., 579 F.3d 1363, 1378 (Fed. Cir. 2009), but they misread it to mean that the "claimed invention" includes unrecited elements. Employing open-ended language does not change the invention; it is simply a way to insure that others cannot avoid infringement by adding to the invention.

If defendants were correct, nearly every open-ended claim would be invalidated. The whole point of such claims is to prevent others from avoiding infringement by adding new elements that the inventors did not anticipate at the time of the invention. If, as the court of appeals has held, patentees are entitled to draft their claims to cover unrecited elements, then it would make no sense to require patentees to explain in the specification how to practice later improvements or additions. Cf. A.B. Dick Co., 713 F.2d at 703 ("[A] pencil

structurally infringing a patent claim would not become noninfringing when incorporated into a complex machine that limits or controls what the pencil can write. Neither would infringement be negated simply because the patentee failed to contemplate use of the pencil in that environment.") (Emphasis added).

Defendants cite two cases to support their argument, but neither of them addresses the question whether a patentee must enable unrecited elements. Rather, both of them involved an applicant that used a broad term in the claim and then failed to explain how to practice the invention with respect to particular aspects of that term. In re Vaeck, 947 F.2d 488, 495 (Fed. Cir. 1991) (affirming patent office's conclusion that claim was not enabled because applicant included "cyanobacteria" element without explaining in specification which cyanobacteria could be used); Sitrick v. Dreamworks, LLC, 516 F.3d 993, 1000 (Fed. Cir. 2008) (claim that disclosed invention related to both movies and video games not enabled because specification did not teach how to practice invention with movies). In the absence of case law requiring the patentee to enable his invention with respect to unrecited elements, I decline to impose such a requirement.

D. Obviousness as to the '235, '598, '660 and '771 patents

The parties agree that all elements of the claims were known in the prior art, with the exception of the particular combinations of loci to be co-amplified. Under 35 U.S.C. §

103(a), a claim is invalid "if the differences between the claimed subject matter and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains." Star Scientific, Inc. v. R.J. Reynolds Tobacco Co., 655 F.3d 1364, 1374 (Fed. Cir. 2011) (internal quotations and alterations omitted). Defendants have the burden to show by clear and convincing evidence that the asserted claims are obvious. Hybritech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1375 (Fed. Cir.1986).

Defendants advance two theories of obviousness. The first is the only theory included in defendants' expert report. It is contingent on defendants' argument that the claims are not enabled unless the specification shows how to practice the inventions using loci not recited in the claims:

In the event that the Promega patents are actually deemed [to] teach and enable skilled artisans to multiplex sets of loci other than those listed in the claims, i.e., arbitrary sets of loci, then the claims would have been obvious in light of the prior art because the prior art would have already taught and enabled the same. Sun Decl., Ex. 8 (Struhl Invalidity Report) ¶ 45. In other words, if trial and error as disclosed in the Promega patents constitutes an enabling disclosure for multiplexing arbitrary sets of loci, then the prior art, which already taught trial and error, would also already have taught multiplexing of arbitrary sets of loci.

Dfts.' Br., dkt. #245, at 44. Because I have rejected defendants' enablement theory, this argument is moot.

Defendants' second theory is that the new loci combinations are not a "significant"

difference from the prior art because "the selection of the number of loci and the specific loci for use in a multiplex is merely an arbitrary choice." Id. at 45-56. This argument suffers from multiple problems. To begin with, it seems to be an afterthought because defendants' expert does not discuss it and defendants have submitted no proposed findings of fact about it. As I noted in the introduction, the court will not consider facts if they are included in a brief but not in the party's proposed findings of fact. Defendants cite <u>United States v. Murphy Oil USA, Inc.</u>, 143 F. Supp. 2d 1054, 1064 (W.D. Wis. 2001), for the proposition that parties should not include legal conclusions in their proposed findings of fact. That is obviously correct, but unhelpful. Expert opinions and descriptions of the prior art are not legal conclusions. In any event, even if I considered the allegations in defendants' brief, defendants cite no evidence showing that it would be obvious to a person of ordinary skill in the art that combinations of loci in the claims can be successfully co-amplified. Because defendants bear the burden of persuasion with respect to invalidity, plaintiff's motion for summary judgment must be granted on the issue of obviousness.

E. Willful Infringement

Finally, plaintiff has moved for summary judgment on the question of willfulness, which it bears the burden to prove by clear and convincing evidence. <u>nCube Corp. v.</u>

Seachange Intern., Inc., 436 F.3d 1317, 1319 (Fed. Cir. 2006). Plaintiff has not shown that

it is entitled to judgment as a matter of law on this issue. "'[W]illful' action is quintessentially a question of fact, for it depends on findings of culpable intent and deliberate or negligent wrongdoing." Biotec Biologische Naturverpackungen GmbH & Co. KG v. Biocorp, Inc., 249 F.3d 1341, 1356 (Fed. Cir. 2001). In fact, plaintiff cites no cases in which a court concluded that the plaintiff was entitled to summary judgment on willfulness. Perhaps more important, plaintiff's argument on willfulness is undeveloped, making up a page of their opening brief and consisting of little more than a few quotations from documents prepared by one employee of defendants. This is insufficient to show as a matter of law that plaintiff is entitled to a finding of willfulness.

ORDER

IT IS ORDERED that

- 1. The motion for partial summary judgment filed by defendants Life Technologies Corporation, Invitrogen IP Holdings, Inc. and Applied Biosystems, LLC, dkt. #234, is GRANTED with respect to plaintiff Promega Corporation's claim of infringement of claims 25 and 27-31 of U.S. Patent No. 5,843,660 and defendants' counterclaims for noninfringement of the same claims. Plaintiff's complaint is DISMISSED as to those claims. Defendants' motion is DENIED in all other respects.
 - 2. Plaintiff's motion for summary judgment, dkt. #227, is GRANTED with respect

to the following claims of infringement:

- AmpFlSTR COfiler PCR Ampliflication Kit infringes claims 23 and 27 of U.S.
 Patent No.6,221,598 and claim 42 of U.S. Patent No. Re 37,984;
- AmpFlSTR Profiler PCR Amplification Kit infringes claims 10, 23-24, 27 and 33 of the '598 patent and claim 42 of the '984 patent;
- AmpFlSTR Identifiler PCR Amplification Kit infringes claims 10, 23-24 and 27 of the '598 patent, claims 18-19 and 21-23 of U.S. Patent No. 6,479,235, claim 5 of U.S. Patent No. 7,008,771 and claim 42 of the '984 patent;
- AmpFISTR Profiler Plus PCR Amplification Kit infringes claim 42 of the '984
 patent; and
- AmpFISTR Yfiler PCR Amplification Kit infringes claim 42 of the '984 patent.

 The motion is DENIED as to all other claims of infringement and inducing infringement.
- 2. Plaintiff's motion for summary judgment, dkt. #227, is GRANTED with respect to defendants' affirmative defenses and counterclaims that the '235, '598, '660 and '771 patents are invalid because they are anticipated, obvious or not enabled. Plaintiff's motion is DENIED with respect to its claim of willfulness.
- 3. Plaintiff's motion to disregard facts not included in the proposed findings of fact, dkt. #262, is GRANTED. Plaintiff's motion for leave to file a reply brief in support of that

motion, dkt. #293, is DENIED as unnecessary.

Entered this 29th day of November, 2011.

BY THE COURT: /s/ BARBARA B. CRABB District Judge Case: 13-1011 CassASE-PARITICIPANTIFSEOTNES DOPARTICIPANTIFSEOTNES DOPARTICIS FIRESTERIO 733 FIRESTERIO 730 FIRESTERIO 740 Z/20 ES ed: 07/12/2013

TAB 2

IN THE UNITED STATES DISTRICT COURT	
FOR THE WESTERN DISTRICT OF WISCONSIN	
PROMEGA CORPORATION,	
Plaintiff,	OPINION and ORDER
and	10-cv-281-bbc
MAX-PLANCK-GESELLSCHAFT zur FORDERUNG der WISSENSCHAFTEN E.V.,	
Involuntary Plaintiff,	
v.	
LIFE TECHNOLOGIES CORPORATION, INVITROGEN IP HOLDINGS, INC. and APPLIED BIOSYSTEMS, LLC,	
Defendants.	
Plaintiff Promage Corneration is suing defendants Life Technologies Corneration	

Plaintiff Promega Corporation is suing defendants Life Technologies Corporation, Applied Biosystems, LLC and Invitrogen IP Holdings, Inc. for infringement of U.S. Patents Nos. 5,843,660, 6,221,598, 6,479,235, 7,008,771 and Re 37,984. Trial is scheduled for February 6, 2012 and the parties' motions in limine are now before the court.

A. Plaintiff's Motions in Limine

1. Motion to preclude references to equitable defenses, dkt. #375

In their answer, defendants raised affirmative defenses and counterclaims for unclean hands, laches and estoppel, but neither side discussed these issues in its summary judgment materials. Plaintiff says that defendants have now waived these counterclaims and defenses because they did not raise them in response to plaintiff's motion for summary judgment. Defendants say they had no obligation to raise those defenses at the time.

The parties debate whether <u>Diversey Lever, Inc. v. Ecolab, Inc.</u>, 191 F.3d 1350 (Fed. Cir. 1999), and <u>Pandrol USA</u>, <u>LP v. Airboss Ry. Products, Inc.</u>, 320 F.3d 1354 (Fed. Cir. 2003), resolve the matter. In <u>Diversey</u>, 191 F.3d at 1352, the court held that the defendant waived an equitable estoppel defense by failing to raise it in response to the plaintiff's motion for summary judgment on "liability." In <u>Pandrol USA</u>, 320 F.3d at 1364, the court held that the defendant did *not* waive an invalidity defense by failing to raise it in response to the plaintiff's motion for summary judgment on "infringement." The key issue in these cases seems to be whether the defendant had notice that the plaintiff's motion for summary judgment required it to come forward with all of its liability defenses or just those related to infringement.

In this case, plaintiff did not limit its motion to "infringement," but it did not say that it was seeking judgment on "liability" either. Rather, it said that it "hereby moves for

summary judgment that certain claims of the Promega Patents and Tautz patent are a) not anticipated, b) not obvious, c) enabled, and d) infringed." Dkt. #228, at 2. The order on summary judgment was limited to those issues; I did not enter judgment in favor of plaintiff on liability generally. Although the question is a close one, I think that plaintiff left enough ambiguity in its motion to preclude a finding of waiver by defendants.

However, that does not resolve plaintiff's motion entirely. The parties agree that equitable defenses are decided by the court, not the jury, so there is no reason that either side should be referring to these defenses in front of the jury. Agfa Corp. v. Creo Products Inc., 451 F.3d 1366, 1375 (Fed Cir. 2006) ("[A]n equitable defense [is] adjudicated by the trial court without a jury."). Defendants object on the ground that plaintiff has not identified any specific evidence to exclude. However, it is not necessary to identify particular testimony or documents at this point. Obviously, if defendants wish to offer evidence that is relevant to one of the issues to be decided by the jury, they may do so even if that evidence happens to be relevant to one of the equitable defenses as well.

Defendants' argument raises another question: what pieces of evidence do defendants intend to offer in support of these defenses? There is no point in holding a court trial on the defenses if defendants have no evidence to support them. Accordingly, I will give defendants an opportunity to identify the grounds for these defenses. If defendants can show that they have support for the defenses, I will hold a court trial after the conclusion of the jury trial.

2. <u>Motion to "preclude references to defendants' arguments on scope of employment and</u> respondeat superior," dkt. #376

This motion relates to Lisa Ortuno, an employee of defendants. Plaintiff argues that defendants should be precluded from arguing at trial that Ortuno was acting outside the scope of her employment with respect to various actions she took that plaintiff says are relevant to its claims for inducement. Defendants say that they do "not intend to assert a scope of employment, or respondeat superior, argument and, therefore, d[o]not intend to introduce evidence or argument regarding that issue," dkt. #442, so this motion will be granted as unopposed.

3. Motion to exclude certain testimony relating to kit sales to universities by damages expert Jonathan Tomlin, dkt. #378

Plaintiff says that Tomlin has improperly excluded from his damages calculation certain sales of defendants' accused kits to universities. First, plaintiff says that Tomlin is wrong to conclude that sales to U.S. universities are covered by a license agreement that extends to "any analysis, based on the measurement of the length of polynucleotide sequence containing a tandem repeat, of human genetic material for (a) use in, or preparation for, legal proceedings, or (b) analysis of biological specimens for the identification of individuals." However, plaintiff simply says that the universities used the kits for "training." It fails to

provide any specific facts about the content of the training or otherwise develop any argument that the training falls outside the scope of the license. Accordingly, I am denying this portion of plaintiff's motion.

Second, with respect to the foreign universities, plaintiff argues that Tomlin did not have enough data to determine the purpose for which those universities were using the kits.

Again, this argument is conclusory. If plaintiff believes that Tomlin's data set is too small to make a generalization, it is free to attempt to establish that through cross-examination.

4. Motion to exclude certain testimony relating to "alternative" and "upper bound" damages, dkt. #380

In his report, plaintiff's damages expert John Beyer set forth three damages estimates that he called "lower bound," "alternative" and "upper bound." Plaintiff wishes to preclude defendants' damages expert Jonathan Tomlin from critiquing Beyer's estimate relating to "alternative" and "upper bound" damages on the ground that Tomlin did not include an analysis of these estimates.

In their response, defendants admit that "Dr. Tomlin does not 'correct' or 'adjust' Dr. Beyer's 'alternative' and 'upper bound' calculations," dkt. #443, at 5, so I will grant plaintiff's motion to the extent that plaintiff's are seeking to preclude Tomlin from offering an adjusted figure. However, Tomlin is free to explain why he believes the jury should reject

Beyer's "alternative" and "upper bound" calculations as a general matter because Tomlin does include that discussion in his report. Dkt. #414, at 6.

5. Motion to "preclude evidence or argument concerning certain terms of the 2006 agreement," dkt. #381

At summary judgment, one of defendants' defenses to infringement was that a 2006 licensing agreement gave them the right to sell accused kits for various purposes: chimerism in the context of bone marrow transplant monitoring, cell line authentication, classifying molar specimens and determinations of fetal sex. I rejected this argument and granted plaintiff's motion for summary judgment for direct infringement of the patents at issue. In its motion, plaintiff asks the court to preclude defendants from relitigating this issue.

In their response, defendants say that they "will not be re-litigating issues already determined by the Court's November 29, 2011 Opinion and Order," but that "there are multiple issues related to the 2006 Cross-License that remain in the case." In particular, defendants say that their understanding of the scope of the license is relevant to inducement and willfulness, that the court has not yet determined whether "forensic training" falls within the scope of the license and that the court has not considered "the specific identity and number of sales that [defendants] sold in unlicensed fields." Dkt. #457. With respect to the third issue, defendants say that they should be able to argue that particular sales of those

kits fall within the license agreement if defendants did not have knowledge of how a customer was going to use a kit.

The first two issues are outside the scope of plaintiff's motion, so I need not consider them. Defendants have waived the third issue. Plaintiff moved for summary judgment on direct infringement with respect to sales of these kits. If defendants believed that the license agreement protected them in instances in which they were unaware of the customer's use of the kit, that is an issue they should have raised in response to plaintiff's motion. Pandrol USA, 320 F.3d at 1366-67; Diversey, 191 F.3d at 1352. Accordingly, I am granting this motion.

6. Motion to preclude defendants from relying on attorney advice as a defense to willfulness, dkt. #382

This motion will be granted as unopposed. Dkt. #444 (Defendants do "not intend to rely on or introduce at trial evidence of attorney advice in defense of willfulness, or any other claim.").

7. Motion to "preclude defendants from introducing evidence or argument on matters decided by claims construction and summary judgment and request for statement to the jury on previous findings," dkt. #383

The only specific issue plaintiff identifies in this motion is a request for an instruction to the jury regarding the issues that have been resolved by the court. Because defendants agree that such an instruction is appropriate, I will grant this portion of the motion. However, plaintiff provides no context for the remainder of the motion, so I cannot decide it at this time.

8. Motion to "preclude testimony on certain fields of use matters prior to 2006 to avoid jury confusion," dkt. #384

I am denying this motion because plaintiff never identifies with any precision what it wants to exclude with this motion or why. Plaintiff refers to the subject matter variously as "evidence prior to 2006 about Promega's inquiries and work in examining clinical diagnostics and other non-permitted fields," "[e]vidence of Promega's actions to commercialize and sell products into particular fields of use prior to 2006" and "matters related to Promega's examination of commercial opportunities." However, plaintiff never explains what it means by this or why such evidence would confuse the jury. Obviously, if defendants wish to use evidence of any events leading up to the 2006 license agreement, they will have to show that the evidence is relevant to the remaining issues for the jury and not an attempt to contradict the summary judgment opinion.

9. Motion to exclude any references to the arbitration proceedings, dkt. #386

This court granted defendants' motion to compel arbitration of a number of claims arising out of a 1996 agreement. Dkt. #140. Because defendants "agre[e] that evidence or testimony relating to the substantive claims that have been referred to arbitration . . . would be irrelevant," dkt. #447, at 2, I am granting this motion as unopposed.

Defendants raise another issue in their response regarding the timing of this lawsuit. In particular, defendants say that "the fact that Promega brought the instant action within one month after service of the Demand for Arbitration under the 1996 License Agreement . . . may be evidence that Promega did not believe that Life was infringing during this period, and accordingly may be evidence relevant to non-willfulness." <u>Id.</u> It is not clear how *plaintiff's* beliefs could be relevant to show *defendants'* intent. In any event, because that issue is beyond the scope of plaintiff's motion, I need not resolve it now.

10. Motion to strike defendants' "newly disclosed" witnesses, dkt. ## 368 and 387

Discovery in this case closed on December 15, 2011. On January 13, 2012, defendants supplemented their Fed. R. Civ. P. 26(a)(1) disclosures with 18 new witnesses. Plaintiff asks the court to strike each of these witnesses as untimely under Fed. R. Civ. P. 37. (Plaintiff says it is seeking to strike 19 witnesses, but defendants point out that plaintiff's list has 18 names on it.) Under Rule 37(c)(1), if a party fails to disclose its evidence as

required under Rule 26, the evidence must be excluded unless the failure was substantially justified or harmless.

Defendants offer two reasons for allowing 13 of the witnesses to testify. (They are not contesting plaintiff's motion with respect to the other five. Dkt. #423, at 6 n.3) First, defendants say that two of the proposed witnesses, Arthur Eisenburg and Guido Sandulli, are not really new because they were identified previously in other discovery. Second, defendants say that the remaining witnesses are necessary to rebut the "wrong assumptions" made by plaintiff's expert John Beyer in a report he filed on December 15.

I am granting this motion because defendants have failed to show that the late supplements were justified or harmless. Simply because a witness's name appears in a discovery document does not mean that the other side has notice that the witness is testifying on a particular topic. To the extent defendants believed that they were unfairly surprised by opinions in Beyer's report, the proper response would have been to file a motion to strike those opinions or seek leave to file a supplemental report from their own expert. With respect to prejudice, because plaintiff had no notice of these witnesses until just before trial, there is no time left for plaintiff to take their depositions or otherwise explore their potential testimony before trial.

As a "sanction," plaintiff asks the court to prohibit any witness from testifying about particular topics. Because plaintiff fails to develop that argument, I am denying this request.

11. Motion to dismiss counts 17, 18 and 19 of the amended complaint without prejudice, dkt #372

This motion is GRANTED as unopposed.

12. Motion to "expand summary judgment ruling to new products," dkt. #373

Plaintiff wants the court to "expand the summary judgment ruling" to include products that were not included in its motion for summary judgment because these products are indistinguishable from those that the court found to be infringing. In response, defendants concede that the following additional products fall within the scope of the summary judgment ruling:

- a. AB Minifiler PCR Amplification Kit (Claim 42 of the '984 Patent);
- b. AB SGM Plus PCR Amplification Kit (Claim 42 of the '984 Patent);
- c. AB SEfiler Kit (Claim 42 of the '984 Patent);
- d. AB SEfiler Plus Kit (Claim 42 of the '984 Patent);
- e. NGM PCR Amplification Kit (1000 and 200) (Claim 42 of the '984 Patent);
- f. NGM SElect Kit (Claim 42 of the '984 Patent);
- g. Identifiler Plus Kit (Claim 42 of the '984 Patent, Claim 5 of the '771
- Patent, Claims 18, 19, 21, 22 and 23 of the '235 Patent, Claims 10, 23, 24,

27, and 33 of the '598 Patent);

h. Identifiler Direct Kit (Claim 42 of the '984 Patent, Claim 5 of the '771

Patent, Claims 18, 19, 21, 22 and 23 of the '235 Patent, Claims 10, 23, 24,

27, and 33 of the '598 Patent);

i. AB Green I PCR Amplification Kit (Claim 42 of the '984 Patent and claims

23 and 27 of the '598 patent1);

j. Blue PCR Amplification Kit (Claim 42 of the '984 Patent); and

k. COfiler + Profiler Plus Kit (Claim 42 of the '984 and claims 23 and 27 of the '598 Patent).

Accordingly, I will amend the summary judgment order to include these additional products with respect to these claims.

B. Defendants' Motions in Limine

1. <u>Motion to exclude testimony of Randall Dimond regarding STR kit use by institution</u> type, dkt. #404

As discussed in the January 31, 2012 order, I am reserving a ruling on this motion to allow the parties to submit supplemental briefs.

2. Motion to exclude "certain testimony" of John Beyer, dkt. #408

Defendants seek to exclude testimony by plaintiff's expert John Beyer on various subjects: (1) the "quantum" of infringing sales by defendant Applied Biosystems; (2) the "interchangeability" of the products of plaintiff and defendants; (3) noninfringing substitutes; (4) plaintiff's manufacturing capacity for STR kits; and (5) demand for plaintiff's products. I will consider each subject in turn.

a. Quantum of infringing sales

This issue overlaps with the motion to exclude certain testimony of Randall Dimond. Again, the question is whether one of plaintiff's experts may provide an opinion about the percentage of defendants' sales that fall outside the scope of the license agreement in the absence of direct evidence on that issue. I will a reserve a ruling on this issue until the parties file their supplemental briefs.

b. "Interchangeability" and non-infringing substitutes

Defendants argue that Beyer is not qualified to testify about the similarity of plaintiff's and defendants' products or the availability of noninfringing substitute products. In its response, plaintiff does not say that Beyer is qualified to give these opinions. Rather, plaintiff says that Beyer was simply parroting the opinions of Dimond on these issues when it was necessary as part of his damages report. Plaintiff points out that defendants do not

challenge that aspect of Dimond's opinions. Accordingly, I will grant this motion, but only to the extent that Beyer's opinion is different from Dimond's.

c. Manufacturing capacity

Again, plaintiff does not argue that Beyer is qualified to give an opinion on plaintiff's manufacturing capacity. Therefore, I am granting this motion to the extent Beyer intends to rely on his own expertise in testifying about this matter.

d. Demand for plaintiff's products

I am denying this motion because defendants fail to develop their argument. They do not deny that Beyer is qualified to testify about product demand, but they say that his opinion relies on "various assumptions" and he "does not consider all the factors" that he should. Dkt. #410, at 43. However, they do not explain what these assumptions are or why his failure to consider individual factors is grounds for excluding his opinion rather than for cross examination.

ORDER

IT IS ORDERED that

1. Plaintiff Promega Corporation's motion to preclude references to equitable

defenses, dkt. #375, is GRANTED IN PART. Defendants Life Technologies Corporation, Applied Biosystems, LLC and Invitrogen IP Holdings, Inc. may not refer to these defenses during the jury trial. Defendants may have until February 6, 2012, to explain in writing the grounds for their equitable defenses and the evidence they have to support those defenses.

- 2. Plaintiff's motion to "preclude references to defendants' arguments on scope of employment and respondeat superior", dkt. #376, is GRANTED as unopposed.
- 3. Plaintiff's motion to exclude certain testimony by damages expert Jonathan Tomlin, dkt. #378, is DENIED.
- 4. Plaintiff's motion to exclude certain testimony relating to "alternative" and "upper bound" damages, dkt. #380, is GRANTED IN PART. Defendants' expert may not provide an adjustment to plaintiff's experts' calculations, but he may challenge the reliability of the calculations.
- 5. Plaintiff's motion to "preclude evidence or argument concerning certain terms of the 2006 agreement," dkt. #381, is GRANTED.
- 6. Plaintiff's motion to preclude defendants from relying on attorney advice as a defense to willfulness, dkt. #382, is GRANTED as unopposed.
- 7. Plaintiff's motion to "preclude defendants from introducing evidence or argument on matters decided by claims construction and summary judgment and request for statement to the jury on previous findings," dkt. #383, is GRANTED IN PART. The court will provide

an instruction to the jury regarding the matters that have been resolved before trial.

- 8. Plaintiff's motion to "preclude testimony on certain fields of use matters prior to 2006 to avoid jury confusion," dkt. #384, is DENIED.
- Plaintiff's motion to exclude any references to the arbitration proceedings, dkt.
 #386, is GRANTED as unopposed.
- 10. Plaintiff's motion to strike defendants' "newly disclosed" witnesses, dkt. ## 368 and 387, is GRANTED. Defendants are precluded from calling the following witnesses at trial, except for impeachment: Phillip Habermeier; Rebecca Clifton; Carla Abdo; Orion Ng; Roberto Castlenovo; Naseem Malik; Beate Balitzki; Katja Anslinger; Franz Neuhuber; Arthur Eisenberg; Solomon F. Ofori-Acquah; Jason Linvelle; Robert Allen; Mary Brophy; Ken Dyu; Steven Wittbrodt; Robert Harris and Graham Consterdine.
- 11. Plaintiff's motion to dismiss counts 17, 18 and 19 of the second amended complaint without prejudice, dkt #372, is GRANTED as unopposed. The second amended complaint is DISMISSED WITHOUT PREJUDICE as to those three counts.
- 12. Plaintiff's motion to "expand summary judgment ruling to new products," dkt. #373, is GRANTED as unopposed. The order dated November 29, 2011, dkt. #345, is AMENDED to grant summary judgment to plaintiff with respect to the following claims of infringement:
 - a. AB Minifiler PCR Amplification Kit infringes claim 42 of the '984 Patent;

- b. AB SGM Plus PCR Amplification Kit infringes claim 42 of the '984 Patent;
- c. AB SEfiler Kit infringes claim 42 of the '984 Patent;
- d. AB SEfiler Plus Kit infringes claim 42 of the '984 Patent;
- e. NGM PCR Amplification Kit (1000 and 200) infringes claim 42 of the '984 Patent;
- f. NGM SElect Kit infringes claim 42 of the '984 Patent;
- g. Identifiler Plus Kit infringes claim 42 of the '984 Patent, claim 5 of the '771 Patent, claims 18, 19, 21, 22 and 23 of the '235 Patent, claims 10, 23, 24, 27 and 33 of the '598 Patent;
- h. Identifiler Direct Kit infringes claim 42 of the '984 Patent, claim 5 of the '771 Patent, claims 18, 19, 21, 22 and 23 of the '235 Patent, claims 10, 23, 24, 27 and 33 of the '598 Patent;
- i. AB Green I PCR Amplification Kit infringes claim 42 of the '984 Patent and claims 23 and 27 of the '598 patent;
- j. Blue PCR Amplification Kit infringes claim 42 of the '984 Patent; andk. COfiler + Profiler Plus Kit infringes claim 42 of the '984 and claims 23 and27 of the '598 Patent.
- 13. Defendants' motion to exclude the testimony of John Beyer is GRANTED IN PART, dkt. #410. Beyer may not offer expert testimony about the similarity of plaintiff's

and defendants' products, the availability of noninfringing substitutes or plaintiff's manufacturing capacity, except to rely on Randall Dimond's opinion. Defendants' motion is DENIED with respect to demand for plaintiff's products.

Entered this 1st day of February, 2012.

BY THE COURT: /s/ BARBARA B. CRABB District Judge Case: 13-1011 CassASB-PARITICIPANTIFSeOtNBS Dorangeen11263 Fileady e071/120/20153ed: 07/12/2013

TAB 3

IN THE UNITED STATES DIST	TRICT COURT	
FOR THE WESTERN DISTRICT	OF WISCONSIN	
PROMEGA CORPORATION,		
Plaintiff,	OPINION and ORDER	
and	10-cv-281-bbc	
MAX-PLANCK-GESELLSCHAFT zur FORDERUNG der WISSENSCHAFTEN E.V.,		
Involuntary Plaintiff,		
V.		
LIFE TECHNOLOGIES CORPORATION, INVITROGEN IP HOLDINGS, INC. and APPLIED BIOSYSTEMS, LLC,		
Defendants.		
In an order dated February 1, 2012, dkt. #486, motions in limine. This order addresses the remaining	, ,	
OPINION		

A. Burden of Proof

One of the main issues for trial will be the extent to which defendants' sales of the

accused products are covered by a 2006 licensing agreement between the parties. A threshold question is which side has the burden of proof. I asked the parties to submit supplemental briefing on this issue so that it could be resolved before trial.

Having reviewed the parties' submissions, I conclude that the burden is properly placed on defendants. The Court of Appeals for the Federal Circuit consistently and repeatedly has described a license as an affirmative defense to a claim for infringement, which the defendant has the burden to prove. Monsanto Co. v. Scruggs, 459 F.3d 1328, 1334 (Fed. Cir. 2006); Chamberlain Group, Inc. v. Skylink Technologies, Inc., 381 F.3d 1178, 1193 (Fed. Cir. 2004); State Contracting & Engineering Corp. v. Condotte America, Inc., 346 F.3d 1057, 1065 (Fed. Cir. 2003); Augustine Medical, Inc. v. Progressive Dynamics, Inc., 194 F.3d 1367, 1370 (Fed. Cir. 1999); In re Cambridge Biotech Corp., 186 F.3d 1356, 1364 (Fed. Cir. 1999); Glass Equipment Development, Inc. v. Besten, Inc., 174 F.3d 1337, 1342 (Fed. Cir. 1999); Carborundum Co. v. Molten Metal Equipment Innovations, Inc., 72 F.3d 872, 878 (Fed. Cir. 1995); Intel Corp. v. US International Trade Commission, 946 F.2d 821, 828 (Fed. Cir. 1991); Met-Coil Systems Corp. v. Korners Unlimited, Inc., 803 F.2d 684, 687 (Fed. Cir. 1986); Bandag, Inc. v. Al Bolser's Tire Stores, Inc., 750 F.2d 903, 924 (Fed. Cir. 1984); Kansas Jack, Inc. v. Kuhn, 719 F.2d 1144, 1148 (Fed. Cir. 1983); Stickle v. Heublein, Inc., 716 F.2d 1550, 1556 (Fed. Cir. 1983). See also Fed. R. Civ. P. 8(c) (listing "license" as affirmative defense). Defendants cite no cases in

which the court of appeals characterized licenses differently or placed the burden on the plaintiff.

Defendants say that the cases discussing licenses relate to the question whether the defendant has the burden to prove the existence of a license rather than its scope. Although many of the cases dealt with the existence of a license, others use more general language. For example, in Spindelfabrik Suessen-Schurr v. Schubert & Salzer Maschinenfabrik Aktiengesellschaft, 903 F.2d 1568, 1576 (Fed. Cir. 1990), the court rejected the defendant's argument that "the district court improperly placed on it the burden of proving that its actions were justified by [a] license." The court reasoned that "[t]he license . . was asserted as a defense to what otherwise would be infringement. As the proponents of the defense, it was incumbent upon [the defendants] to show that the license authorized the sale of the infringing machines in the United States." See also Rockwell International Corp. v. United States, 31 Fed. Cl. 70, 77 (Fed. Cl. 1994) ("[D]efendant has the burden of proof to show the existence of a license and the scope of any such license."); Technical Development Corp. v. United States, 597 F.2d 733, 746 (Ct. Cl. 1979) ("The defendant bears the burden of proof on the license defense."). In addition, plaintiff cites several district court cases in which the court placed the burden on the defendant to prove a license defense. A. Natterman & Cie Gmbh v. Bayer Corp., 428 F. Supp. 2d 253, 258 (E.D. Pa. 2006); Ciena Corp., v. Corvis Corp., 334 F. Supp. 2d 610, 613 (D. Del. 2004); Cyrix Corp., v. Intel Corp.,

846 F. Supp. 522, 535 (E.D. Tex. 1994); <u>CIVIX-DDI, LLC v. Nat'l Ass'n of Realtors</u>, Case No. 05 C 6869, 2006 WL 3210504 (N.D. Ill. Nov. 6, 2006). Defendants cite no contrary authority. In fact, defendants acknowledge a case in which the Supreme Court stated, "[i]f a licensee be sued, he can escape liability to the patentee for the use of his invention by showing that the use is within his license." <u>De Forest Radio Telephone & Telegraph Co. v.</u> United States, 273 U.S. 236, 242 (1927).

In any event, defendants identify no persuasive reason for distinguishing between the burden with respect to the existence of a license and its scope. They cite no other examples in which the defendant has the burden to prove certain aspects of an affirmative defense, but the plaintiff has the burden with respect to others. The point of an affirmative defense is that the defendant has the burden to prove that it is entitled to the defense.

In support of their argument that plaintiff should have the burden of proof, defendants rely almost entirely on language in 35 U.S.C. § 271(a) that makes it unlawful to use a patented invention "without authority." However, defendants cites no authority for the proposition that the phrase in § 271(a) places the burden of proof on plaintiff and they fail to reconcile their argument with the many cases in which courts have characterized a license as an affirmative defense to infringement. If it were the plaintiff's burden to show that the defendant acted "without authority" to practice a patent, then it would also be the plaintiff's burden to show that no license existed, whether express or implied.

Further, it simply makes sense to put the burden on defendants to show that their uses of the patent are protected by the license because they have the best access to that information. Metzl v. Leininger, 57 F.3d 618, 622 (7th Cir. 1995) ("Economy in litigation also requires that burdens of presenting evidence be assigned to the parties that can produce the necessary evidence at least cost."). See also Green Tree Financial Corp.-Alabama v. Randolph, 531 U.S. 79, 96 (2000) ("where fairness so requires, burden of proof of a particular fact may be assigned to 'party who presumably has peculiar means of knowledge' of the fact") (quoting 9 J. Wigmore, Evidence § 2486 (J. Chadbourn rev. ed.1981)). It was defendants, not plaintiff, who determined how defendants would sell their products. Placing the burden on the patent owner would require the owner to undergo intensive investigations to police the license and would give infringers an incentive to keep poor records or no records on their sales in an attempt to hide their infringing activities. If defendants have the burden, they have every incentive to keep their records detailed and clear.

Defendants devote much of their brief to citing cases in which courts have stated that the plaintiff has "ha[s] the burden to prove the extent to which the infringing method has been used." <u>Lucent Technologies, Inc. v. Gateway, Inc.</u>, 580 F.3d 1301, 1334-35 (Fed. Cir. 2009). This is true but irrelevant. Of course, plaintiff will have the burden to prove which sales are *infringing*, but that is a different question from which sales fall within the scope of the license. Although I understand defendants' wish to conflate the two questions, I believe

they are separate. Once plaintiff has proven that defendants sold an infringing product or induced a third party to practice the patented method, it will be defendants' burden to show that a particular sale was protected by the license agreement.

B. Plaintiff's Motions

1. Motion for leave to file supplemental report of John Beyer, dkt. #463

Plaintiff seeks leave to supplement Beyer's report to account for data that defendants recently produced. Defendants do not oppose this request, so I will grant it. Plaintiff asks for sanctions as well, but I am denying that request because plaintiff failed to make any showing that defendants violated Rule 26 or a court order. In addition, I am denying defendants' request in their response brief to file a rebuttal to this new report because they have failed to show that this request is justified.

2. Motion to strike supplemental report of Jonathan Tomlin, dkt. ## 436 and 450

It is undisputed that defendants produced Tomlin's supplemental report several weeks after the close of discovery and the night before his deposition, in violation of this court's scheduling order. Defendants justify the untimely report on the grounds that it was "submitted only in response to a rebuttal report submitted by Dr. John Beyer on the last day of discovery" and that plaintiff was given an "unrestricted opportunity to depose Dr. Tomlin

about his supplemental report."

Defendants' first argument is a nonsequitur. As I explained in the order granting plaintiff's motion to strike 18 of defendants' witnesses for untimeliness, "[t]o the extent defendants believed that they were unfairly surprised by opinions in Beyer's report, the proper response would have been to file a motion to strike those opinions or seek leave to file a supplemental report from their own expert." Dkt. #486, at 10. Defendants did neither and it is too late to seek permission to file a report now.

Defendants' second argument is not much better. Although plaintiff was able to depose Tomlin, this was less than 24 hours after receiving the report, leaving plaintiff little time to prepare. As defendants acknowledge, this court's scheduling order required the parties to provide expert reports at least five days in advance of the deposition. Defendants do not even attempt to explain why they failed to comply with that requirement. If I accepted defendants' argument that plaintiff was not prejudiced simply because it had the opportunity to depose Tomlin, this would encourage parties to sandbag their opponents with last minute supplemental reports just before a deposition. Because defendants have not shown that the late report was justified or harmless, I am granting this motion.

C. <u>Defendants' Motions</u>

1. Motion to exclude testimony of Randall Dimond regarding STR kit use by institution

type, dkt. #404

I reserved a ruling on this motion in the February 1 order to allow the parties to file supplemental briefs on the burden of proof issue. Now that I have resolved that issue in favor of plaintiff, this motion becomes less important, but I agree with defendants that it is not necessarily moot if plaintiff still intends to rely on this opinion.

In his report, plaintiff's expert Randall Dimond offers the opinion that "the nature of each institution permits one to infer how the kits will be employed." Dkt. #316, at 25. He then goes on to list the ways various types of institutions, such as hospitals and universities, "would be expected" to use the accused products. Id. at 26-29. Defendants do not argue that it is inappropriate as a general matter to use expert testimony this way, but they say that the opinion should be excluded because Dimond "does not disclose any methodology that he employed to reach his conclusions." Dkt. #404, at 4.

Under Fed. R. Evid. 702, the court must decide whether the proffered expert testimony is "based upon sufficient facts or data" and is "the product of reliable principles and methods." Although Dimond could have done a better job explaining exactly how he reached his conclusions, it is clear enough from his various expert reports that he was relying on his extensive personal experience working with these different institutions and his review of the literature regarding the institutions to support his opinions. That is enough to allow Dimond to testify. Defendants are free to present contrary evidence or challenge Dimond's

testimony on cross examination.

2. Motion to exclude "certain testimony" of John Beyer, dkt. #408

I resolved several parts of this motion in the February 1, order, but I reserved a ruling with respect to Beyer's opinions on defendants' "quantum of infringing sales." Again, because I have concluded that defendants should have the burden of proof regarding the sales that were permitted by the license, Beyer's opinion on this issue may be less important, but I will resolve the motion to the extent plaintiff intends to rely on this testimony.

Beyer set forth three damages estimates that he called "lower bound," "alternative" and "upper bound." Defendants devoted 40 pages of their brief to challenging Beyer's opinion on this issue, which is longer than Beyer's entire expert report. Defendants raise many specific objections to the opinion, but they fall into two general categories: (1) Beyer is not qualified to testify about how a particular customer uses a kit; and (2) Beyer fails to explain how he generated any of his sales estimates.

Plaintiff's response brief is just as lengthy as defendants' motion, but plaintiff fails to respond directly to most of defendants' criticisms. With respect to the first criticism, plaintiff says that Beyer relied on Dimond's opinions to make determinations that required any expertise. However, it is impossible to tell from either of Beyer's reports the extent to which he relied on Dimond. With respect to the second criticism, plaintiff does not try to

defend Beyer's reports. Instead, it relies heavily on a declaration that Beyer prepared with plaintiff's brief in opposition to defendants' motion to strike. By calling this document a "declaration," plaintiff tries to obscure the fact that it is really an untimely supplemental report. I cannot hold defendants accountable for filing late supplemental reports, but look the other way when plaintiff does the same. Because Beyer fails to explain in his reports how he arrived at his "upper bound," "lower bound" or "alternative" estimates, I must grant defendants' motion to strike this aspect of his opinion.

ORDER

IT IS ORDERED that

- 1. Plaintiff Promega Corporation's motion for leave to file a supplemental report of John Beyer, dkt. #463, is GRANTED.
- 2. Plaintiff's motion to strike the supplemental report of Jonathan Tomlin, dkt. ## 436 and 450, is GRANTED.
- 3. The motion filed by defendants Life Technologies Corporation, Applied Biosystems, LLC and Invitrogen IP Holdings, Inc. to exclude testimony of Randall Dimond regarding STR kit use by institution type, dkt. #404, is DENIED.
 - 4. Defendants' motion to exclude the testimony of John Beyer related to his "upper

bound," "lower bound" and "alternative" estimates, dkt. #408, is GRANTED.

Entered this 3d day of February, 2012.

BY THE COURT: /s/ BARBARA B. CRABB District Judge Case: 13-1011 Cast ASE-PARITICIPANTISEO IN BY DORANGE 11383 FIREO 11382/20 F3 ed: 07/12/2013

TAB 4

1 UNITED STATES DISTRICT COURT FOR THE WESTERN DISTRICT OF WISCONSIN 2 PROMEGA CORPORATION, 3 Plaintiff, and 4 MAX-PLANCK-GESELLSCHAFT zur FORDERUNG der WISSENSCHAFTEN E.V., 5 Involuntary Plaintiff, -vs-Case No. 10-CV-281-BBC Madison, Wisconsin 6 LIFE TECHNOLOGIES CORPORATION, INVITROGEN IP HOLDINGS INC. February 6, 2012 7 and APPLIED BIOSYSTEMS, LLC, 3:00 p.m. Defendants. 8 STENOGRAPHIC TRANSCRIPT OF FIRST DAY OF JURY TRIAL 9 AFTERNOON SESSION HELD BEFORE DISTRICT JUDGE BARBARA B. CRABB, and a jury, 10 APPEARANCES: For the Plaintiff: Troupis Law Office, LLC 11 ATTORNEYS JAMES TROUPIS, STEWART KARGE and PETER CARROLL 12 8500 Greenway Blvd., Ste. 200 Middleton, Wisconsin 53562 13 Also present: William Linton, CEO of Promega Craig Christenson, General Counsel 14 RMR, CRR, CBC Lynette Swenson Federal Court Reporter 15 U.S. District Court 120 N. Henry St., Rm. 520 Madison, WI 53703 (608) 255-3821 1-P-2 16

1	acceptable. We simply want, when we do find out how
2	they summarize that, for us to be able to analyze it and
3	him to be able to respond if that is, in fact, what we
4	want to do.
5	MS. JOHNSON: Your Honor, as I understood
6	Dr. Beyer's opinions in his reports and his deposition,
7	he was relying on alleged issues with the data to
8	support his bound methodology. So certainly to the
9	extent that he is trying to use that evidence for that
10	purpose, I think that would subvert the Court's order.
11	THE COURT: I'm not so if he doesn't use it
12	he's not going to talk about the bounds anymore.
13	MS. JOHNSON: Correct. Yes. That would
14	certainly be our position.
15	THE COURT: Okay. All right. Then as far as
16	whether the research, educational and training uses of
17	the kits are covered by the license, do you wish to say
18	anything further about that?
19	MR. WIKSTROM: I do, Your Honor. The basic
20	problem we have is would be lack of fair notice. By
21	the plaintiffs' own admission, Dr. Dimond did some
22	research before this litigation even began and according
23	to them was aware of some research reports that he
24	thought were outside the bounds of the license. As I
25	

Т	Michelle Shepherd back on July 26th, and in that
2	deposition they specifically asked her about
3	institutions, some universities in this country, and she
4	testified that they were doing forensic teaching,
5	training, testing, and knowing that, when they moved for
6	summary judgment on infringement, they said nothing
7	about it. They only identified the uses that you
8	identified in your order. And so basically what that
9	would do to us, it would be putting the burden on us to
10	defend a use that we didn't know at the time they were
11	even contending was that they were challenging; we
12	didn't know that they were contending was outside of the
13	license. And so how they can now say that we waived it
14	is beyond me.
15	We took the position in summary judgment that all
16	of our uses fell within the definition and all they came
17	back with were the specific uses that you found. And so
18	and then they started honing in on it just before
19	discovery closed and it's simply too late in the day.
20	Their entire brief focuses on the language of "for
21	use in the identification of individuals." But as Your
22	Honor knows from reading our brief, the primary claim we
23	make is that these uses are an integral part of
24	developmental and internal validation, which at the very
25	least would be preparation for use of the kits and the

products of these kits in court proceedings.
I think the legal framework that you look at on
this is very important as well because California law
applies, and California law is a little bit different.
Under California law, you don't have to find an
ambiguity before the Court is required to look at
extrinsic evidence of interpretation and also how it's
viewed in the industry. And the California Court of
Appeals cases that the plaintiffs cite simply don't
overrule the California Supreme Court case; that I think
was the Proctor & Gamble case that established this line
of authority that we rely on. And so the interpretation
that they're advancing would really eviscerate the
license that we thought we purchased from them back in
2006 because if you can't train people to use our kits,
then they're not qualified to testify about those kits
if they ever go in a court proceeding. If you can't
and not only have to be trained initially, but the FBI
guidelines that apply to this require them to
demonstrate they have to be certificated initially and
then they have to be they have to establish their
proficiency twice a year, and the only way you can do
that is by using these kits.
The research that plaintiffs cited in their brief
is also an integral part of what the standards that

1	govern all of this called developmental validation. For
2	example the first study that is cited in their brief by
3	Dr
4	THE COURT: And I've read all this
5	Mr. Wikstrom.
6	MR. WIKSTROM: I know you've read it, Your
7	Honor, but are you aware that this first study was done
8	by the SPSA, which stands for the Scottish Police
9	Service Authority, which is the forensic the crime
10	Scottish Forensic Crime Lab? And what they're doing in
11	that study is they're characterizing genetic markers for
12	internal validation purposes. And I can tell you on
13	each one of these studies that they've cited how it
14	relates to validating a method of that is used; a
15	necessary part of the testimony. You have to validate
16	your DNA collection methods, one of those deals with
17	cheek swabs; you have to validate the if you're going
18	to bring another product into the procedure along with
19	the kits, you have to validate the ability to use that
20	in the process or you can't testify. People are always
21	looking for ways to poke holes in the DNA evidence and
22	so this science has to be incredibly rigorous, and so
23	that's what the research is and mostly developmental
24	validation and internal validation.
25	The reason that we say that it would eviscerate our

1	license is that ultimately if we couldn't do those
2	things, pretty soon our kits fall off we can't sell
3	our kits because we can't validate them; we can't
4	validate the people that use them. For those reasons,
5	Your Honor, we've always understood, until very recently
6	in this case, that forensic research, training and
7	education were part of the forensics license that we
8	got.
9	It also so happens that when the students actually
10	use the kits or the people use the kits in training and
11	validation, they're using them the kits exactly the
12	way the kits were designed to be used. The fact that
13	they're testing themselves against a known standard;
14	i.e., somebody knows who that individual is, the
15	students don't necessarily, is not the determining
16	factor. Because if that was the case, you can imagine a
17	prosecution for the crime of rape where the accused is
18	actually caught at the scene and then they to
19	corroborate, they do a DNA testing of the bodily fluids.
20	Under their interpretation of the license, our kits
21	couldn't be used because the police knew the identity of
22	the person they caught at the scene. Yet they're doing
23	the test as part of the criminal process.
24	THE COURT: I think that's taking it a little
25	bit far, but I don't think the waiver issue is important

LifeTechPromega

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1	and I'm not deciding it on the basis of waiver. I think
2	it's just obvious that research, educational and
3	training is not covered by the license unless it's done
4	right there in the police department. But you're
5	talking about genetic research that's done at
6	universities that are not connected with any law
7	enforcement agencies. And as helpful as that may be to
8	some sort of identification purpose down the line, that
9	is not what's covered by the license.
10	When I this was first broached, I had an idea
11	that this was going to be used for new people working at
12	the crime lab who were going to be okay, these were
13	the kits we use; here is how we use them; this is what
14	we do. But when I saw the declarations that were
15	presented and realized that this applied or at least
16	that defendants wanted it to apply to every research
17	project going on in the world that had anything to do
18	with genetics, no. No. Doesn't work.
19	All right. Then anything else to discuss before
20	tomorrow morning?
21	MR. TROUPIS: Yes. Your Honor, the deposition
22	designations, which while they won't be coming up
23	tomorrow, they'll actually be coming up the following
24	day, ought to be addressed at this point. We followed
25	Your Honor's suggestion Friday and the deposition

Case: 13-1011 Cast ASE-PARITICIPANTIFS ENTINES DO Caugne 11:463 Filter of 27/14/2/2013

TAB 5

IN THE UNITED STATES DISTRICT COURT FOR THE WESTERN DISTRICT OF WISCONSIN

PROMEGA CORPORATION,

Plaintiff.

AMENDED
JUDGMENT IN A CIVIL CASE

and

Case No. 10-cv-281-bbc

MAX-PLANCK-GESELLSCHAFT zur FORDERUNG der WISSENSCHAFTEN E.V.,

Involuntary Plaintiff,

V.

LIFE TECHNOLOGIES CORPORATION, INVITROGEN IP HOLDINGS, INC. and APPLIED BIOSYSTEMS, LLC,

Defendants.

This action came for consideration before the court with District Judge Barbara B. Crabb presiding. The issues have been considered and a decision has been rendered.

IT IS ORDERED AND ADJUDGED that judgment is entered:

- (1) granting defendants' motion for partial summary judgment with respect to plaintiff's claim of infringement of claims 25 and 27-31 of U.S. Patent No. 5,843,660 and defendants' counterclaims for non-infringement of the same claims;
- (2) granting plaintiff's motion for summary judgment with respect to defendants' counterclaims that U.S. Patent Nos. 6,479,235, 6,221,598, 5,843,660 and 7,008,771 are invalid because they are anticipated, obvious or not enabled;
- (3) dismissing the counterclaims filed by defendants for their failure to prove these counterclaims; and

1	damont	-	-	Cini	10	in

Page 2

(4) granting defendants' motion for judgment as a matter of law regarding 35 U.S.C.

§ 271(a) and (f)(1).

Approved as to form this 14th day of September, 2012.

Barbara B. Crabb, District Judge

9/18/12 Date

Case: 13-1011 Cast ASE-PARITICIPANTISEO IN BY DOR augment 14933 Filter of the OT/142/2013

TAB 6



US005843660A

Patent Number:

United States Patent [19]

Schumm et al. [45] Date of Patent: Dec. 1, 1998

[11]

[54] MULTIPLEX AMPLIFICATION OF SHORT TANDEM REPEAT LOCI

[75] Inventors: James W. Schumm, Madison; Katherine A. Micka, Oregon; Dawn R.

Rabbach, DeForest, all of Wis.

[73] Assignee: Promega Corporation, Madison, Wis.

[21] Appl. No.: 632,575

[22] Filed: Apr. 15, 1996

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 316,544, Sep. 30, 1994.

[51] **Int. Cl.**⁶ **C12Q 1/68**; C12P 19/34; C07H 21/04

[52] **U.S. Cl.** **435/6**; 435/91.2; 536/24.33; 935/77; 935/78

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Primary Examiner—Stephanie W. Zitomer Attorney, Agent, or Firm—Grady J. Frenchick; Stroud, Stroud, Willink, Thompson & Howard; Karen B. King

[57] ABSTRACT

The present invention is directed to the simultaneous amplification of multiple distinct genetic loci using PCR or other amplification systems to determine in one reaction the alleles of each of the loci contained within the multiplex.

43 Claims, 19 Drawing Sheets

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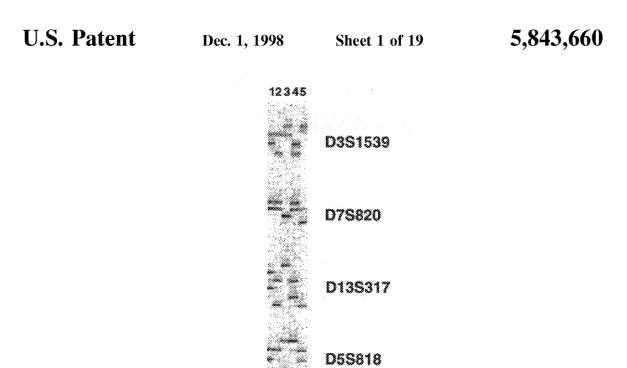
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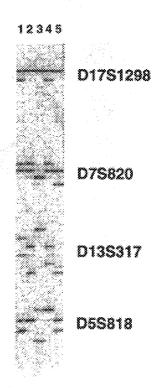


FIGURE 2

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D17S1298



D7S820



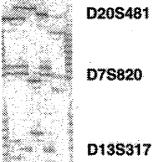
D13S317



D5S818

FIGURE 3





D 100017

D5S818

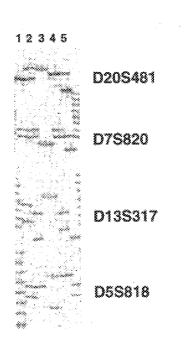
FIGURE 4



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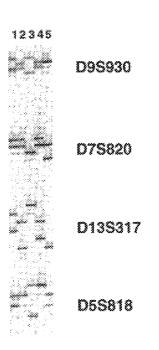
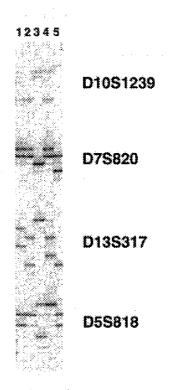


FIGURE 6

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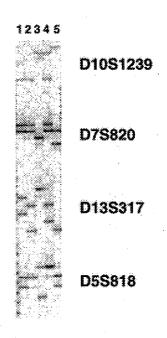
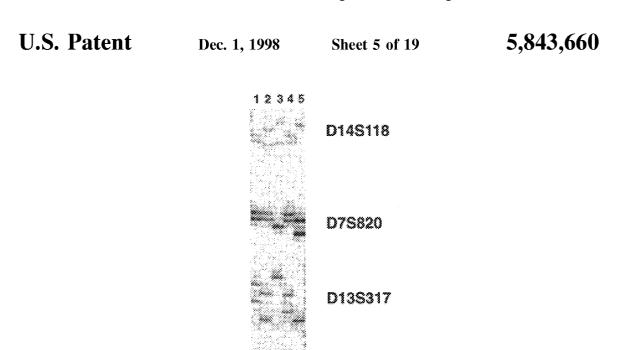


FIGURE 8



D5S818

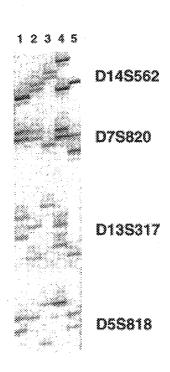


FIGURE 10

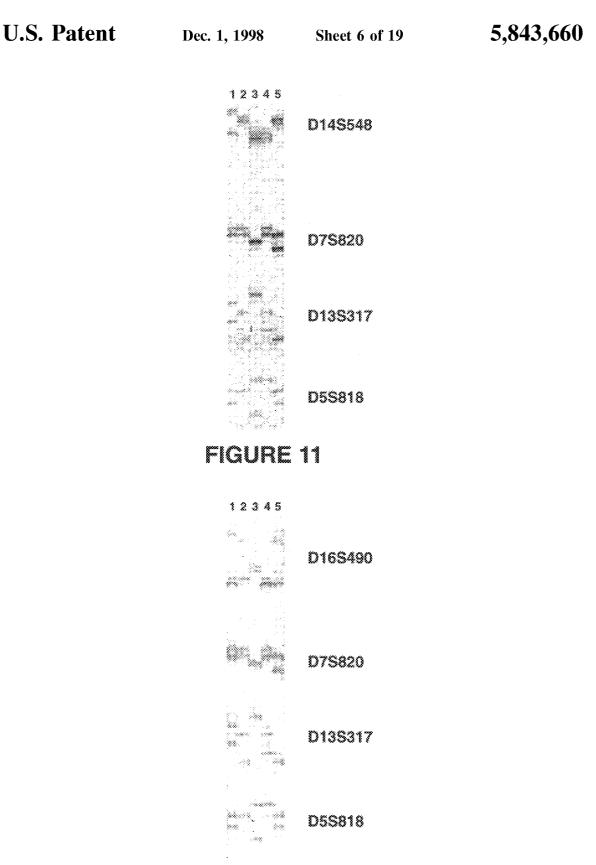


FIGURE 12

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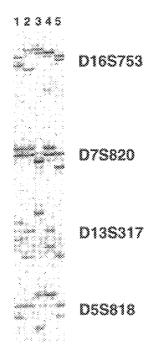


FIGURE 13

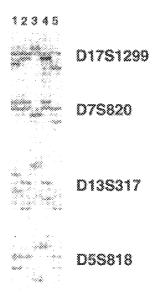


FIGURE 14

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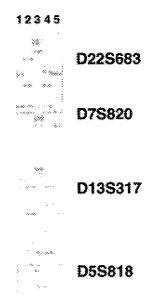


FIGURE 16

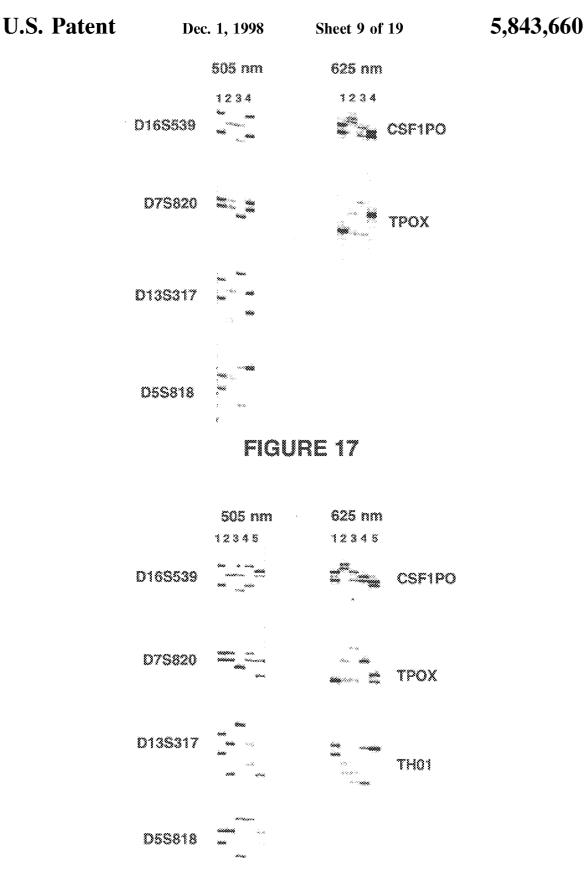


FIGURE 18

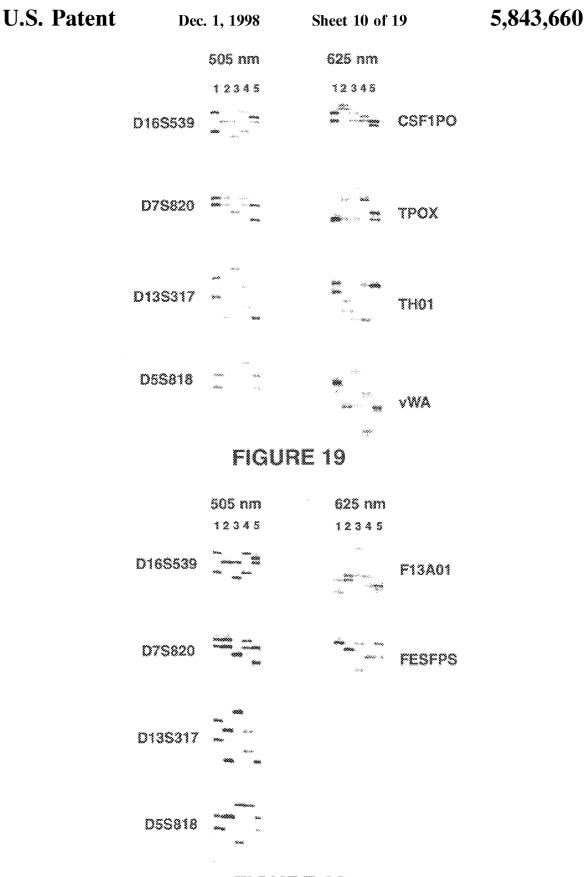


FIGURE 20

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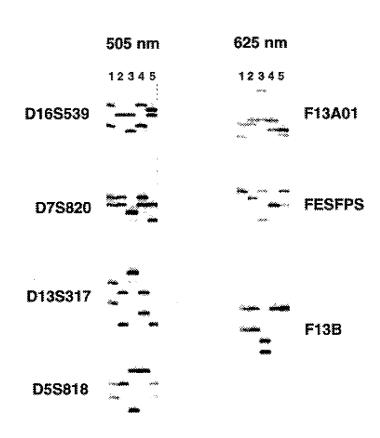


FIGURE 21

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U.S. Patent

505 nm 625 nm 12345 12345 D16\$539 F13A01 D7S820 FESFPS D13S317 F13B D5S818

FIGURE 22

LPL

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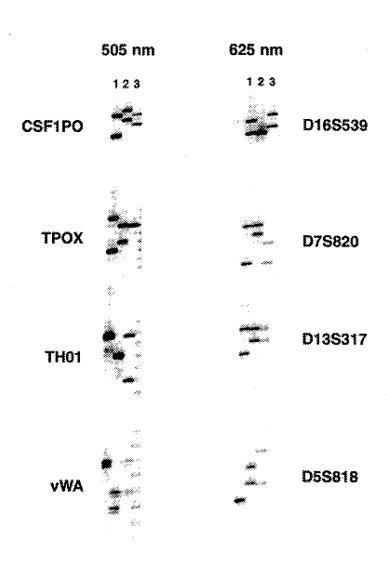
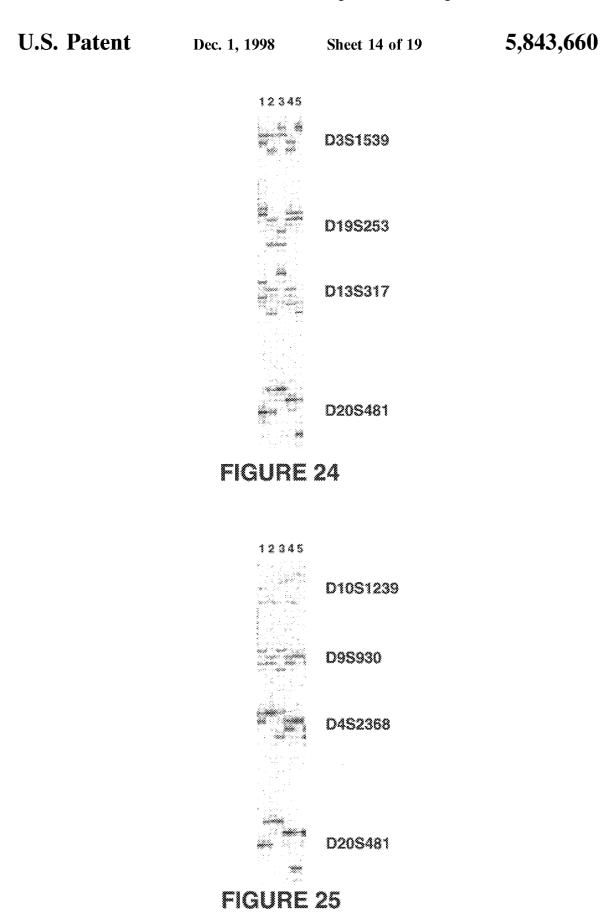
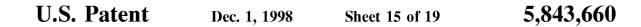


FIGURE 23





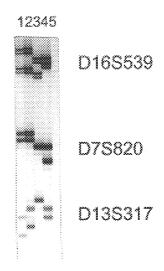


FIGURE 26

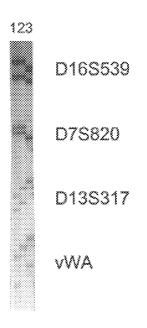


FIGURE 27

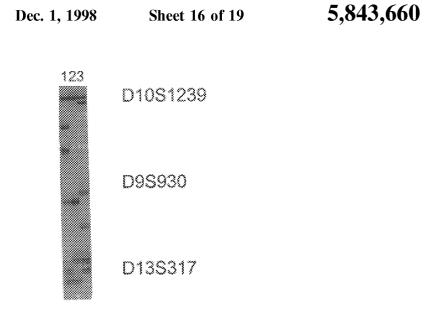


FIGURE 28

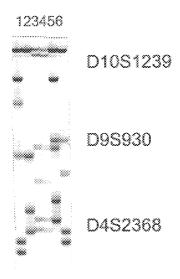
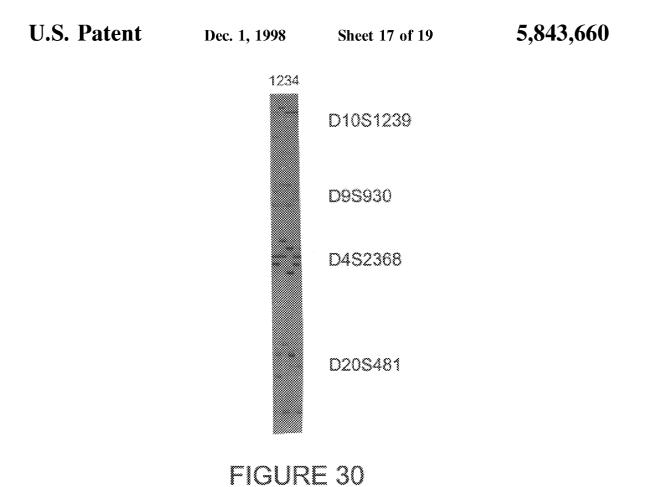


FIGURE 29



D3S1539
D19S253
D13S317

FIGURE 31

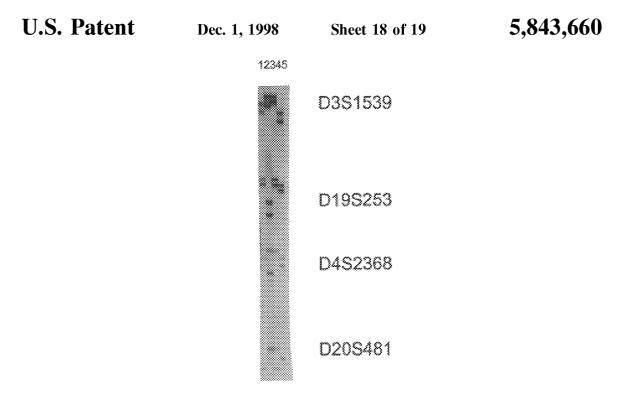


FIGURE 32

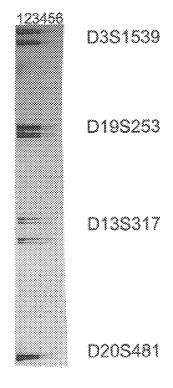
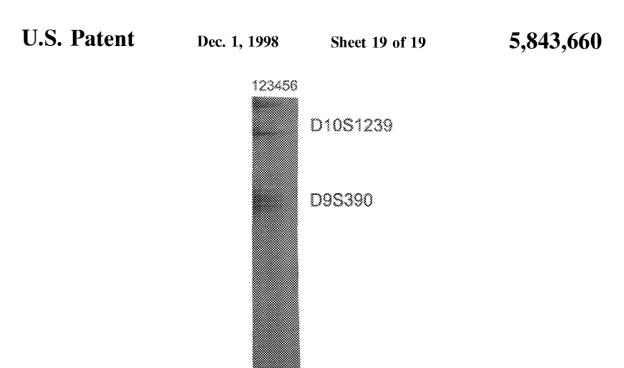
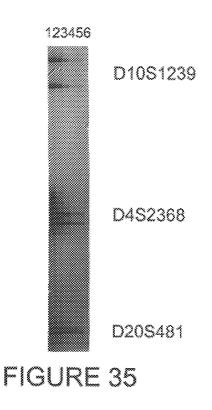


FIGURE 33



D20S481

FIGURE 34



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MULTIPLEX AMPLIFICATION OF SHORT TANDEM REPEAT LOCI

This application is a continuation-in-part of U.S. patent application Ser. No. 08/316,544, filed Sep. 30, 1994. The 5 entire disclosure of that parent application is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention is generally directed to the detection of genetic markers in a genomic system. The present invention is more specifically directed to the simultaneous amplification of multiple distinct polymorphic genetic loci using the polymerase chain reaction or other amplification systems to determine in one reaction the alleles of each locus contained within the multiplex system.

BACKGROUND OF THE INVENTION

In recent years, the discovery and development of poly- $_{\rm 20}$ morphic short tandem repeats (STRs) as genetic markers has stimulated progress in the development of linkage maps, the identification and characterization of diseased genes, and the simplification and precision of DNA typing.

Many loci, at least in the human genome, contain poly- 25 morphic STR regions (Adamson, D., et al. (1995) "A collection of ordered tetranucleotide-repeat markers from the human genome," Am. J. Hum. Genet. 57: 619–628; Murray, J. C., et al. (1994) "A comprehensive human linkage Hudson, T. J., Engelstein, M., Lee, M. K., Ho, E. C., Rubenfield, M. J., Adams, C. P., Housman, D. E., and Dracopoli, N. C. (1992) "Isolation and chromosomal assignment of 100 highly informative human simple sequence repeat polymorphisms," Genomics 13: 622-629). STR loci consist of short, repetitive sequence elements of 3 to 7 base pairs in length. It is estimated that there are 2,000,000 expected trimeric and tetrameric STRs present as frequently as once every 15 kilobases (kb) in the human genome with trimeric and tetrameric tandem repeats." Am. J. Hum. Genet. 49: 746–756; Beckman, J. S., and Weber, J. L. (1992) "Survey of human and rat microsatellites," Genomics 12: 627-631). Nearly half of the STR loci studied by Edwards of genetic markers.

Variation in the number of short tandem repeat units at a particular locus causes the length of the DNA at that locus to vary from allele to allele and from individual to individual. Such length polymorphism is reminiscent of variable 50 number of tandem repeats (VNTR) loci (Nakamura, Y., et al. (1987) "Variable number of tandem repeat (VNTR) markers for human gene mapping," Science 235: 1616-1622) and minisatellite loci (Jeffreys, A. J., et al. (1985) "Hypervariable 'minisatellite' regions in human DNA," Nature 314: 67-73), both of which contain considerably longer repeat units than STR loci. Such length polymorphism is also reminiscent of the dinucleotide repeat form of microsatellite loci (Litt, M. and Luty, J. A. (1989) "A hypervariable microsatellite revealed by in-vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene," Am. J. Hum. Genet. 44: 397-401, Tautz, D., et al. (1986) "Cryptic simplicity in DNA is a major source of genetic variation," Nature 322: 652-656, Weber, J. L. and May, P. E. (1989) "Abundant class of human DNA polymorphisms 65 which can be typed using the polymerase chain reaction," Am. J. Hum. Genet. 44: 388-396; Beckmann and Weber,

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(1992)), a form of microsatellite loci with shorter repeat units than STR loci.

Polymorphic STR loci are extremely useful markers for human identification, paternity testing and genetic mapping. STR loci may be amplified via the polymerase chain reaction (PCR) by employing specific primer sequences identified in the regions flanking the tandem repeat.

Alleles of these loci are differentiated by the number of copies of the repeat sequence contained within the amplified region and are distinguished from one another following electrophoretic separation by any suitable detection method including radioactivity, fluorescence, silver stain, and color.

To minimize labor, materials and analysis time, it is desirable to analyze multiple loci and/or more samples 15 simultaneously. One approach for reaching this goal involves amplification of multiple loci simultaneously in a single reaction. Such "multiplex" amplifications, as they are called, have been described extensively in the literature. Multiplex amplification sets have been extensively developed for analysis of genes related to human genetic diseases such as Duchenne Muscular Dystrophy (Chamberlain, J. S., et al. (1988) "Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification," Nucleic Acid Res. 16: 11141–11156; Chamberlain, J. S., et al. (1989), "Multiple PCR for the diagnosis of Duchenne muscular dystrophy," In PCR Protocols, A Guide to Methods and Application (ed. Gelfand, D. H., et al.) pp. 272-281. Academic Press, San Diego, Calif.; Beggs, A. H., et al. (1990) "Detection of 98% DMD/BMD gene deletions by PCR," map with centimorgan density," Science 265: 2049-2054; 30 Hum. Genet. 86: 45-48; Clemens, P. R., et al. (1991). "Carrier detection and prenatal diagnosis in Duchenne and Becker muscular dystrophy families, using dinucleotide repeat polymorphisms," Am J. Hum. Genet. 49: 951-960; Schwartz, J. S., et al. (1992) "Fluorescent multiple linkage analysis and carrier detection for Duchenne/Becker's muscular dystrophy," Am J. Hum. Genet. 51: 721-729; Covone, A. E., et al. (1992) "Screening Duchenne and Becker muscular dystrophy patients for deletions in 30 exons of the dystrophin gene by three-multiplex PCR," Am. J. Hum. (Edwards et al. (1991) "DNA typing and genetic mapping 40 Genet. 51: 675-677), Lesch-Nyhan Syndrome (Gibbs, R. A., et al. (1990) "Multiple DNA deletion detection and exon sequencing of the hypoxanthine phosphoribosyltransferase gene in Lesch-Nyhan families," *Genomics* 7: 235–244), Cystic Fibrosis (Estivill, X., et al. (1991) "Prenatal diagnosis et al. (1991) are polymorphic, which provides a rich source 45 of cystic fibrosis by multiplex PCR of mutation and microsatellite alleles," Lancet 338: 458; Fortina, P., et al. (1992) "Non-radioactive detection of the most common mutations in the cystic fibrosis transmembrane conductance regulator gene by multiplex polymerase chain reaction," Hum. Genet. 90: 375-378; Ferrie, R. M., et al. (1992) "Development, multiplexing, and application of ARMS tests for common mutations in the CFTR gene," Am. J. Hum. Genet. 51: 251-262; Morral, N. and Estivill, X. (1992) "Multiplex PCR amplification of three microsatellites within the CFTR gene," Genomics 51: 1362-1364), and Retinoblasma (Lohmann, D., et al. (1992) "Detection of small RB1 gene deletions in retinoblastoma by multiplex PCR and highresolution gel electrophoresis," Hum. Genet. 89: 49-53). Multiplex amplification of polymorphic microsatellite markers (Clemens et al. (1991); Schwartz et al. (1992); Huang, T. H.-M., et al. (1992) "Genetic mapping of four dinucleotide repeat loci DXS435, DXS45, DXS454, DXS424, on the X chromosome using the multiplex polymerase chain reaction," Genomics 13: 375-380) and even STR markers (Edwards, A., et al. (1992) "Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups," Genomics 12: 241-253; Kimpton, C. P., et al.

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(1993) "Automated DNA profiling employing multiplex amplification of short tandem repeat loci," PCR Methods and Applications 3: 13-22; Hammond, H. A., et al. (1994) "Evaluation of 13 STR loci for use in personal identification applications," Am. J. Hum. Genet. 55: 175-189; Schumm, J. 5 W. et al. (1994) "Development of nonisotopic multiplex amplification sets for analysis of polymorphic STR loci," in "The Fourth International Symposium on Human Identification 1993," pp. 177-187; Oldroyd, N. J., et al. (1995) "A highly discriminating octoplex short tandem repeat poly- 10 reveals the polymorphic nature of the systems employed. merase chain reaction system suitable for human individual identification," Electrophoresis 16: 334-337) have been described.

These amplified products are generally separated by one of several methods of electrophoresis known to those skilled 15 in the art. Several well-known methods of detection of the amplified products have also been described. While ethidium bromide staining or silver staining of amplified fragments is employed in some cases, in others it is preferred to use methods which label only one of the two strands of the 20 amplified material. Examples of this include radioactive or fluorescent labeling of one of the two primers prior to the amplification of a locus. One of the more sophisticated approaches to detection is the use of different fluorescent labels to allow detection of amplified materials representing 25 different loci, but existing in the same space following electrophoresis. The products of the different loci are differentiated with the use of filters or other discriminating detectors, which allow visualization of one fluorescent label at a time.

Reference is made to International Publications WO 93/18177 and WO 93/18178 to Fortina et al., which are directed to methods and kits for diagnosing diseases such as Cystic Fibrosis and β-thalassemia, respectively, using an allele-specific multiplex polymerase chain reaction system. According to Fortina et al., multiplex PCR has also been used for simultaneous amplification of multiple target sequences, permitting mutant allele scanning using two lanes of an agarose gel.

Ballabio, A. et al. (1991) "PCR Tests for Cystic Fibrosis Deletion," Nature, 343: 220, disclose a single-tube, multiplex allele-specific PCR test using two different dye-tagged fluorescent primers for detection of the F508 cystic fibrosis mutation.

While there are multiplex amplification procedures for specific loci, the use of multiplex amplification procedures is greatly desired for the detection of alleles in other types of loci such as specific STR loci. It is also desirable to identify primers which make multiplex amplification of such 50 loci possible.

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide a method and materials for the simultaneous ampli- 55 fication of multiple distinct polymorphic short tandem repeat (STR) loci using PCR or other amplification systems to determine, in one reaction, the alleles of each locus contained within the multiplex. Multiplex analysis of the sets of specific STR loci disclosed herein have not been previously described in the prior art. There has also not been any previous description of the sequences for many of the primers disclosed herein below, all of which are shown to be useful for multiplex amplification of such STR loci.

It is also an object of the present invention to provide a 65 method, a kit, and primers specific for multiplex amplifications comprising specified loci.

These and other objects are addressed by the present invention which is directed to a method and materials for simultaneously analyzing or determining the alleles present at each individual locus of each multiplex. In general, the method of this invention comprises the steps of (a) obtaining at least one DNA sample to be analyzed, wherein the DNA sample has at least two loci which can be amplified together; (b) amplifying the STR sequences in the DNA sample; and (c) detecting the amplified materials in a fashion which

More specifically, the method of this invention is a method of simultaneously determining the alleles present in at least three tandem repeat loci from one or more DNA samples, such method comprising the steps of:

- (a) obtaining at least one DNA sample to be analyzed, wherein the DNA sample has a set of at least three loci which can be amplified together, wherein the set of loci is selected from a specific group or sets of loci disclosed herein:
- (b) co-amplifying the set of loci in a multiplex amplification reaction, wherein the product of the reaction is a mixture of amplified alleles from each of the co-amplified loci in the set; and
- (c) evaluating the amplified alleles in the mixture to determine the alleles present at each of the loci analyzed in the set within the DNA sample.

In one embodiment of this invention, three STR loci are amplified together, and the set of three co-amplified loci is 30 selected from the group of sets consisting of:

D3S1539, D19S253, D13S317;

D10S1239, D9S930, D20S481;

D10S1239, D4S2368, D20S481;

D10S1239, D9S930, D4S2368;

D16S539, D7S820, D13S317; and

D10S1239, D9S930, D13S317.

In a more preferred embodiment of the method of this invention, the DNA sample has at least four STR loci which 40 can be amplified together, and the set of co-amplified loci is selected from the group of loci consisting of:

D3S1539, D4S2368, D5S818, D7S820, D9S930, D10S1239,

D13S317, D14S118, D14S548, D14S562, D16S490, D16S539,

D16S753, D17S1298, D17S1299, D19S253, D20S481, D22S683,

HUMCSF1PO, HUMTPOX, HUMTH01, HUMFESFPS, HUMF13A01,

HUMBFXIII, HUMLIPOL, HUMvWFA31.

The set of at least four STR loci amplified together is more preferably a set selected from the sets of four loci comprising:

D3S1539, D7S820, D13S317, D5S818; D17S1298, D7S820, D13S317, D5S818; D20S481, D7S820, D13S317, D5S818; D9S930, D7S820, D13S317, D5S818; D10S1239, D7S820, D13S317, D5S818; D14S118, D7S820, D13S317, D5S818; D14S562, D7S820, D13S317, D5S818; D14S548, D7S820, D13S317, D5S818; D16S490, D7S820, D13S317, D5S818; D17S1299, D7S820, D13S317, D5S818; D16S539, D7S820, D13S317, D5S818;

D22S683, D7S820, D13S317, D5S818; D16S753, D7S820, D13S317, D5S818;

D3S1539, D19S253, D13S317, D20S481;

D3S1539, D19S253, D4S2368, D20S481;

D10S1239, D9S930, D4S2368, D20S481; and

D16S539, D7S820, D13S317, HUMvWFA31.

More preferably, the set of STR loci amplified together is a set of six loci, selected from the sets of loci comprising:

D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, 10 HUMTPOX; and

D16S539, D7S820, D13S317, D5S818, HUMF13A01, HUMFESFPS.

Yet more preferably, the set of STR loci amplified together is a set of seven loci, selected from the sets of loci com-

D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, HUMTPOX, HUMTH01; and

D16S539, D7S820, D13S317, D5S818, HUMF13A01, $_{20}$ HUMFESFPS, HUMBFXIII.

Even more preferably, the set of STR loci amplified together is a set of eight loci, selected from the sets of loci compris-

D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, 25 HUMTPOX, HUMTH01, HUMvWFA31; and

D16S539, D7S820, D13S317, D5S818, HUMF13A01, HUMFESFPS, HUMBFXIII, HUMLIPOL.

The multiplex amplification reaction step of the method is preferably done using a pair of primers flanking each locus 30 preferably evaluated by comparing the amplified alleles to a in the set of loci co-amplified in the reaction. More preferably, pairs of primers are selected for the multiplex amplification reaction which produce alleles from each locus that do not overlap the alleles of the other loci in the set co-amplified therein, when the alleles are separated by gel 35 electrophoresis. Even more preferably, the sequence of one of each pair of primers used in the multiplex amplification reaction is selected from a group of primer sequences

SEQ ID NO:1 and SEQ ID NO:2, when one of the loci in 40 the set is D7S820;

SEQ ID NO:3 and SEQ ID NO:4, when one of the loci in the set is D13S317;

SEQ ID NO:5 and SEQ ID NO:6, when one of the loci in the set is D5S818;

SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:49, when one of the loci in the set is D3S1539;

SEQ ID NO:9, SEQ ID NO:10, when one of the loci in the set is D17S1298:

SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:52, SEQ ID NO:53, when one of the loci in the set is D20S481;

SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:55, SEQ ID NO:61, when one of the loci in the set is D9S930;

SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:54, when 55 one of the loci in the set is D10S1239;

SEQ ID NO:17, SEQ ID NO:18, when one of the loci in the set is D14S118;

SEQ ID NO:19, SEQ ID NO:20, when one of the loci in $_{60}$ the set is D14S562;

SEQ ID NO:21, SEQ ID NO:22, when one of the loci in the set is D14S548;

SEQ ID NO:23, SEQ ID NO:24, when one of the loci in the set is D16S490;

SEQ ID NO:25, SEQ ID NO:26, when one of the loci in the set is D16S753;

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SEQ ID NO:27, SEQ ID NO:28, when one of the loci in the set is D17S1299;

SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:58, when one of the loci in the set is D16S539;

SEQ ID NO:31, SEQ ID NO:32, when one of the loci in the set is D22S683;

SEQ ID NO:33, SEQ ID NO:34, when one of the loci in the set is HUMCSF1PO;

SEQ ID NO:35, SEQ ID NO:36, when one of the loci in the set is HUMTPOX;

SEQ ID NO:37, SEQ ID NO:38, when one of the loci in the set is HUMTH01;

SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:59, SEQ ID NO:60 when one of the loci in the set is HUMvWFA31;

SEQ ID NO:41, SEQ ID NO:42, when one of the loci in the set is HUMF13A01;

SEQ ID NO:43, SEQ ID NO:44, when one of the loci in the set is HUMFESFPS;

SEQ ID NO:45, SEQ ID NO:46, when one of the loci in the set is HUMBFXIII;

SEQ ID NO:47, SEQ ID NO:48, when one of the loci in the set is HUMLIPOL;

SEQ ID NO:50, SEQ ID NO:51, when one of the loci in the set is D19S253; and

SEQ ID NO:56, SEQ ID NO:57, when one of the loci in the set is D4S2368.

In the method of this invention, the amplified alleles are size standard, wherein the size standard is selected from the group of size standards consisting of a DNA marker and a locus-specific allelic ladder. The evaluation of alleles is preferably done using polyacrylamide gel electrophoresis to separate the alleles, thereby forming a polyacrylamide gel of separated alleles. The separated alleles in the polyacrylamide gel are preferably determined by visualizing the alleles with an appropriate technique such as silver staining, but more preferably with fluorescent analysis.

Fluorescent analysis is preferably done by labeling one primer of each pair of primers used in the multiplex amplification reaction with a fluorescent label prior to use in the reaction. The fluorescent label used to label each such primer is preferably a fluorescein label or a tretramethyl 45 rhodamine label. Most preferably, at least two different labels are used to label the different primers which are used in the multiplex amplification reaction.

The at least one DNA sample to be analyzed using the method of this invention is preferably isolated from human 50 tissue, preferably tissue selected from the group consisting of blood, semen, vaginal cells, hair, saliva, urine, bone, buccal samples, amniotic fluid containing placental cells or fetal cells, and mixtures of any of the tissues listed above.

In an alternative embodiment, the invention is a kit for simultaneously analyzing STR sequences in at least three loci, the kit comprising a container which has oligonucleotide primer pairs for co-amplifying a set of at least three short tandem repeat loci, wherein the set of loci are selected from the sets of loci consisting of:

D3S1539, D19S253, D13S317;

D10S1239, D9S930, D20S481;

D10S1239, D4S2368, D20S481;

D10S1239, D9S930, D4S2368;

D16S539, D7S820, D13S317;

D10S1239, D9S930, D13S317;

D3S1539, D7S820, D13S317, D5S818;

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D17S1298, D7S820, D13S317, D5S818; D20S481, D7S820, D13S317, D5S818; D9S930, D7S820, D13S317, D5S818; D10S1239, D7S820, D13S317, D5S818; D14S118, D7S820, D13S317, D5S818; D14S562, D7S820, D13S317, D5S818; D14S548, D7S820, D13S317, D5S818; D16S490, D7S820, D13S317, D5S818; D17S1299, D7S820, D13S317, D5S818; D16S539, D7S820, D13S317, D5S818; D22S683, D7S820, D13S317, D5S818; D16S753, D7S820, D13S317, D5S818; D3S1539, D19S253, D13S317, D20S481; D3S1539, D19S253, D4S2368, D20S481; D10S1239, D9S930, D4S2368, D20S481; D16S539, D7S820, D13S317, HUMvWFA31; D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, ₂₀ **HUMTPOX**; D16S539, D7S820, D13S317, D5S818, HUMF13A01,

HUMFESFPS:

D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, HUMTPOX, HUMTH01;

D16S539, D7S820, D13S317, D5S818, HUMF13A01, HUMFESFPS, HUMBFXIII;

D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, HUMTPOX, HUMTH01, HUMvWFA31; and

D16S539, D7S820, D13S317, D5S818, HUMF13A01, 30 HUMFESFPS, HUMBFXIII, HUMLIPOL.

At least one of the primers in each primer pair included in the kit preferably has a sequence selected from one of the groups of sequences listed under the description of the method of this invention, above.

In yet a third embodiment, the invention is primer sequences and primer pairs for amplifying specific STR loci of human DNA. Use of the primers and primer pairs of this invention for multiplex analysis of human DNA is demonstrated herein below. The primers of this invention are 40 suitable for use in the method of this invention, wherein they can be used in either labeled or unlabeled form depending, as noted above, upon how the amplified alleles are to be determined in the evaluating step of the method.

The present invention, in all its various embodiments 45 described briefly above, provides a high throughput method and materials for the detection and analysis of polymorphic genetic markers using specific combinations of loci and specified conditions. By selection of the appropriate detection technique for the evaluation step, the materials and 50 method of this invention can be used in laboratories which have only a power supply and a standard apparatus for polyacrylamide gel electrophoresis or those which have the latest in equipment for fluorescent gel scanning, e.g., FluorImager™ 575 (Molecular Dynamics, Sunnyvale, Calif.) or 55 the Hitachi FMBIO™ (San Bruno, Calif.) fluorescent scanners or the ABI 373 and ABI Prism™ 377 DNA Sequencers (Applied Biosystems Division, Perkin Elmer, Foster City, Calif.). Thus, the method of the present invention is adaptable for a variety of uses and laboratories.

The approach as specified in the present invention produces a savings in time, labor and materials in the analysis of loci contained within the multiplexes. The method of the present invention allows three or more, even as many as eight or more, loci to be amplified together in one tube using 65 (Molecular Dynamics, Sunnyvale, Calif.) in Example 2. a single amplification reaction, instead of amplifying each locus independently in separate tubes.

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The present invention has specific use in the field of forensic analysis, paternity determination, monitoring of bone marrow transplantation, linkage mapping, and detection of genetic diseases and cancers. By allowing three or more loci to be amplified and analyzed simultaneously, the materials and methods of the present invention significantly increase the certainty with which one can match DNA isolated from the blood or other tissues of two different individuals. The need to distinguish accurately between 10 small amounts of tissue of different individuals is particularly acute in forensics applications, where many convictions (and acquittals) turn on DNA typing analysis, including the analysis of STR loci.

Scientists, particularly forensic scientists, have long 15 appreciated the need to analyze multiple polymorphic loci of DNA in order to ensure that a match between two samples of tissue is statistically significant. (Presley, L. A. et al. (1993) "The implementation of the polymerase chain reaction (PCR) HLA DQ alpha typing by the FBI laboratory," in "The Third International Symposium on Human Identification 1992," pp. 245-269; Bever, R. A., et al. (1992) "Characterization of five VNTR loci by Hae III RFLP analysis: application to paternity testing," in "The Second International Symposium on Human Identification 1991," pp. 103-128.) However, until this invention, there were few ways one could simultaneously analyze three or more STR loci in a single reaction. To realize the importance of such multiplexing capabilities, it helps to understand some of the mathematics behind DNA typing analysis.

For purposes of illustration, suppose every STR locus has a genotype (i.e., pattern of two alleles) frequency of one in ten. In other words, suppose that the chance of two randomly selected individuals have a matching type for a single STR is 1/10. However, if two different STR loci are analyzed, the chance of a random match with both systems becomes 1/100. If three STR loci are analyzed, the chances of a random match with each of the three systems become 1/1,000 and so on. Consequently, it is easy to see how increasing the number of STR loci analyzed to any number of loci over three significantly reduces the likelihood of random matches within the general population, thereby increasing the chance one can accurately identify (or eliminate) a suspect in a crime by comparing his type with crime scene evidence. Similar reasoning can be used to conclude that the method of this invention also would increase the likelihood of accurately identifying a suspected father in a paternity case, of correctly matching bone marrow tissue, of developing significant results from linkage mapping studies, and of detecting genetic diseases and cancers.

Further objects, features, and advantages of the invention will be apparent from the following detailed description of the invention and the illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D3S1539, D7S820, D13S317, and D5S818 as detected with a FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 1.

FIG. 2 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D17S1298, D7S820, D13S317, and D5S818 as detected with a FluorImager™ fluorescent scanner

FIG. 3 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of

the loci D17S1298, D7S820, D13S317, and D5S818 as detected with a FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 3.

FIG. 4 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of ⁵ the loci D20S481, D7S820, D13S317, and D5S818 as detected with a FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 4.

FIG. 5 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D20S481, D7S820, D13S317, and D5S818 as detected with a FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 5.

FIG. 6 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D9S930, D7S820, D13S317, and D5S818 as detected with a FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 6.

FIG. 7 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D10S1239, D7S820, D13S317, and D5S818 as detected with a FluorImagerTM fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.)in Example 8.

FIG. 8 is a laser-printed image showing the fluorescent 25 detection of the products of simultaneous amplification of the loci D10S1239, D7S820, D13S317, and D5S818 as detected with a FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 8.

FIG. 9 is a laser-printed image showing the fluorescent 30 detection of the products of simultaneous amplification of the loci D14S118, D7S820, D13S317, and D5S818 as detected with a FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 9.

FIG. 10 is a laser-printed image showing the fluorescent ³⁵ detection of the products of simultaneous amplification of the loci D14S562, D7S820, D13S317, and D5S818 as detected with a FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 10.

FIG. 11 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D14S548, D7S820, D13S317, and D5S818 as detected with a FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 11.

FIG. 12 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D16S490, D7S820, D13S317, and D5S818 as detected with a FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 12.

FIG. 13 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D16S753, D7S820, D13S317, and D5S818 as detected with a FluorImager™ T fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 13.

FIG. 14 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D17S1299, D7S820, D13S317, and D5S818 as detected with a FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 14.

FIG. 15 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D16S539, D7S820, D13S317, and D5S818 as detected with a FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 15.

FIG. 16 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of

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the loci D22S683, D7S820, D13S317, and D5S818 as detected with a FluorImager[™] fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 16.

FIG. 17 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D16S539, D7S820, D13S317, D5S818, HUMCSF1PO ("CSF1PO") and HUMTPOX ("TPOX") as detected with an FMBIO™ Fluorescent Scanner™ (Hitachi Software Engineering, San Bruno, Calif.) in Example 17.

FIG. 18 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D16S539, D7S820, D13S317, D5S818, HUMCSF1PO ("CSF1PO"), HUMTPOX ("TPOX") and HUMTH01 ("TH01") as detected with an FMBIO™ Fluorescent Scanner™ (Hitachi Software Engineering, San Bruno, Calif.) in Example 18.

FIG. 19 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D16S539, D7S820, D13S317, D5S818, HUMCSF1PO ("CSF1PO"), HUMTPOX ("TPOX"), HUMTH01 ("TH01") and HUMvWFA31 ("vWA") as detected with an FMBIO™ Fluorescent Scanner™ (Hitachi Software Engineering, San Bruno, Calif.) in Example 19.

FIG. **20** is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D16S539, D7S820, D13S317, D5S818, HUMF13A01 ("F13A01") and HUMFESFPS ("FESFPS") as detected with an FMBIO™ Fluorescent Scanner™ (Hitachi Software Engineering, San Bruno, Calif.) in Example 20.

FIG. 21 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D16S539, D7S820, D13S317, D5S818, HUMF13A01 ("F13A01"), HUMFESFPS ("FESFPS") and HUMBFXIII ("F13B") as detected with an FMBIO™ Fluorescent Scanner™ (Hitachi Software Engineering, San Bruno, Calif.) in Example 21.

FIG. 22 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D16S539, D7S820, D13S317, D5S818, HUMF13A01 ("F13A01"), HUMFESFPS ("FESFPS"), HUMBFXIII ("F13B") and HUMLIPOL ("LPL") as detected with an FMBIO™ Fluorescent Scanner™ (Hitachi Software Engineering, San Bruno, Calif.) in Example 22.

FIG. 23 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D16S539, D7S820, D13S317, D5S818, HUMCSF1PO ("CSF1PO"), HUMTPOX ("TPOX"), HUMTH01 ("TH01") and HUMvWFA31 ("vWA") as detected with an FMBIO™ Fluorescent Scanner™ (Hitachi Software Engineering, San Bruno, Calif.) in Example 23.

FIG. 24 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D3S1539, D19S253, D13S317 and D20S481 as detected with a FluorImager[™] fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 24.

FIG. 25 is a laser-printed image showing the fluorescent detection of the products of simultaneous amplification of the loci D10S1239, D9S930, D4S2368 and D20S481 as detected with a FluorImager[™] fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.) in Example 25.

FIG. **26** is a photograph illustrating the silver stain detection of the products of simultaneous amplification of three loci, D16S539, D7S820, and D13S317, in Example 26.

FIG. 27 is a photograph illustrating the silver stain detection of the products of simultaneous amplification of four

loci, D16S539, D7S820, D13S317 and HUMvWFA31 ("vWA"), in Example 27.

FIG. 28 is a photograph illustrating the silver stain detection of the products of simultaneous amplification of three loci, D10S1239, D9S930, and D13S317, in Example 28.

FIG. 29 is a photograph illustrating the silver stain detection of the products of simultaneous amplification of three loci, D10S1239, D9S930, and D4S2368, in Example 29.

FIG. 30 is a photograph illustrating the silver stain detection of the products of simultaneous amplification of four 10 loci, D10S1239, D9S930, D4S2368 and D20S481, in Example 30.

FIG. 31 is a photograph illustrating the silver stain detection of the products of simultaneous amplification of three loci, D3S1539, D19S253 and D13S317, in Example 31.

FIG. 32 is a photograph illustrating the silver stain detection of the products of simultaneous amplification of four loci, D3S1539, D19S253, D4S2368 and D20S481, in Example 32.

FIG. 33 is a photograph illustrating the silver stain detection of the products of simultaneous amplification of four loci, D3S1539, D19S253, D13S317 and D20S481, in Example 33.

FIG. 34 is a photograph illustrating the silver stain detection of the products of simultaneous amplification of three loci, D10S1239, D9S930 and D20S481, in Example 34.

FIG. 35 is a photograph illustrating the silver stain detection of the products of simultaneous amplification of three loci, D10S1239, D4S2368 and D20S481, in Example 35.

DETAILED DESCRIPTION OF THE **INVENTION**

A. Definitions

The following definitions are intended to assist in providing a clear and consistent understanding of the scope and 35 detail of the terms:

Allelic ladder: a standard size marker consisting of amplified alleles from the locus.

Allele: a genetic variation associated with a segment of sequence occupying the same locus.

Biochemical nomenclature: standard biochemical nomenclature is used herein in which the nucleotide bases are designated as adenine (A); thymine (T); guanine (G); and cytosine (C). Corresponding nucleotides are, for example, 45 reaction. deoxyguanosine-5'-triphosphate (dGTP).

DNA polymorphism: the condition in which two or more different nucleotide sequences in a DNA sequence coexist in the same interbreeding population.

Locus (or genetic locus): a specific position on a chro- 50 mosome. Alleles of a locus are located at identical sites on homologous chromosomes.

Locus-specific primer: a primer that specifically hybridizes with a portion of the stated locus or its complementary strand, at least for one allele of the locus, and does not 55 hybridize efficiently with other DNA sequences under the conditions used in the amplification method.

Polymerase chain reaction (PCR): a technique in which cycles of denaturation, annealing with primer, and extension with DNA polymerase are used to amplify the number of copies of a target DNA sequence by approximately 106 times or more. The polymerase chain reaction process for amplifying nucleic acid is covered by U. S. Pat. Nos. 4,683,195 and 4,683,202, which are incorporated herein by reference for a description of the process.

Polymorphic short tandem repeat loci: STR loci in which the number of repetitive sequence elements (and net length 12

of sequence) in a particular region of genomic DNA varies from allele to allele, and from individual to individual.

Polymorphism information content (PIC): a measure of the amount of polymorphism present at a locus (Botstein et al., 1980). PIC values range from 0 to 1.0, with higher values indicating greater degrees of polymorphism. This measure generally displays smaller values than the other commonly used measure, i.e., heterozygosity. For markers that are highly informative (heterozygosities exceeding about 70%), the difference between heterozygosity and PIC is slight.

Primary reaction: initial reaction using the purified human genomic DNA as template for the PCR.

Primers: two single-stranded oligonucleotides or DNA fragments which hybridize with opposing strands of a locus 15 such that the 3' termini of the primers are in closest proximity.

Primer pair: two primers including, primer 1 that hybridizes to a single strand at one end of the DNA sequence to be amplified and primer 2 that hybridizes with the other end on the complementary strand of the DNA sequence to be amplified.

Primer site: the area of the target DNA to which a primer hybridizes.

Secondary reaction: reamplification with the same or different primer pair using a dilution of the primary reaction as template for the PCR.

Short tandem repeat loci (STR loci): regions of the human genome which contain short, repetitive sequence elements of 3 to 7 base pairs in length.

30 B. Selection of Multiplex Reaction Components

The method of the present invention contemplates selecting an appropriate set of loci, primers, and amplification protocols to generate amplified alleles from multiple co-amplified loci which either do not overlap in size or which are labeled in some way to make the amplified alleles which do overlap in size distinguishable from one another. In addition, this method contemplates the selection of short tandem repeat loci which are compatible for use with a single amplification protocol. The specific combinations of DNA, i.e., one of two or more alternate forms of a DNA 40 loci described herein are unique in this application. Combinations of loci may be rejected for either of the above two reasons, or because, in combination, one or more of the loci do not produce adequate product yield, or fragments which do not represent authentic alleles are produced in this

> Successful combinations in addition to those disclosed herein can be generated by trial and error of locus combinations, by selection of primer pair sequences, and by adjustment of primer concentrations to identify an equilibrium in which all included loci may be amplified. Once the method and materials of this invention are disclosed, various methods of selecting loci, primer pairs, and amplification techniques for use in the method and kit of this invention are likely to be suggested to one skilled in the art. All such methods are intended to be within the scope of the appended claims

> Of particular importance in the practice of the method of this invention is the size range of amplified alleles produced from the individual loci which are amplified together in the multiplex amplification reaction step. For ease of analysis with current technologies, systems which can be detected by amplification of fragments smaller than 500 bases are most preferable. The most preferable combinations of loci, primers, and amplification techniques are described in the Summary of the Invention section, above.

> Inappropriate selection of primers can produce several undesirable effects such as lack of amplification, amplifica-

tion at multiple sites, primer dimer formation, undesirable interaction of primer sequences from different loci, production of alleles from one locus which overlap with alleles from another, or the need for amplification conditions or protocols for the different loci which are incompatible in a 5 multiplex. Synthesis of the primers used in the present method can be conducted using any standard procedure for oligonucleotide synthesis known to those skilled in the art. C. Use of Multiplexes of Three Loci to Develop Multiplexes Using More than Three Loci

Any one of a number of different techniques can be used to select the set of STR loci to be analyzed using a method of the present invention. One preferred technique for developing useful sets of loci for use in this method of analysis is described below. Once a multiplex containing three loci is 15 of beta-globin genomic sequences and restriction site analydeveloped, it may be used as a core to create multiplexes containing more than three loci. New combinations are created including the first three loci. For example, the core multiplex containing loci D7S820, D13S317, and D5S818 was used to generate derivative multiplexes of D16S539, 20 D7S820, D13S317, and D5S818; HUMCSF1PO, HUMTPOX, D16S539, D7S820, D13S317, and D5S818; HUMCSF1PO, HUMTPOX, HUMTH01, D16S539, D7S820, D13S317, and D5S818; and HUMCSF1PO, HUMTPOX, HUMTH01, HUMvWFA31, D16S539, 25 D7S820, D13S317, and D5S818.

It is contemplated that core sets of loci can be used to generate other appropriate derivative sets of STR loci for multiplex analysis using the method of this invention. Regardless of what method is used to select the loci analyzed using the method of the present invention, all the loci selected for multiplex analysis should share the following characteristics: (1) they should produce minimal slippage (e.g., from misreading the repeat sequence during an amplification step), (2) few if any artifacts due to the addition or 35 deletion of a base to the amplified alleles during the multiplex amplification step, (3) few if any artifacts due to premature termination of amplification reactions by a polymerase, and (4) no "trailing" bands of smaller molecular weight from consecutive single base deletions below a given 40 authentic amplified allele. See, e.g., Schumm et al., "Development of Nonisotopic Multiplex Amplification Sets for Analysis of Polymorphic STR Loci," Fourth International Symposium on Human Identification 1993, pp. 177-187 (pub. by Promega Corp., 1993).

D. Preparation of DNA Samples

Samples of human genomic DNA can be prepared for use in the method of this invention using any method of DNA preparation which is compatible with the amplification of a single locus. Many such methods are suitable for use in 50 preparing genomic DNA samples for use in the method of this invention, including, but not limited to, the methods of DNA sample preparation described by Patel, P. I., et al. (1984) "Organization of the HPRT gene and related sequences in the human genome," Somat Cell Mol Genet 10: 55 483-493, and Gill, P., et al. (1985) "Forensic application of DNA 'fingerprints'," Nature 318: 577-579.

DNA concentrations can be measured prior to use in the method of the present invention, using any standard method of DNA detection. However, the DNA concentration is preferably measured fluorometrically using a measurement technique such as that described by Brunk C. F., et al. 4 (1979) "Assay for nanogram quantities of DNA in cellular homogenates," Anal Biochem 92: 497-500. The DNA concentration is more preferably measured by comparison of the 65 amount of hybridization of DNA standards with a humanspecific probe such as that described by Waye et al. (1979)

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Waye, J. S., et al. (1991) "Sensitive and specific quantification of human genomic deoxyribonucleic acid (DNA) in forensic science specimens: casework examples," J. Forensic Sci., 36:1198–1203. Use of too much template DNA in the amplification reactions can produce artifacts which appear as extra bands which do not represent true alleles. E. Amplification of DNA

Once a sample of human genomic DNA is isolated, and its concentration determined as described above, the targeted 10 loci can be co-amplified in the multiplex amplification step of the present method. Any one of a number of different amplification methods can be used to amplify the loci, including, but not limited to, polymerase chain reaction (PCR) (Saiki, R. K., et al. (1985) "Enzymatic amplification sis for diagnosis of sickle cell anemia," Science 230: 1350–1354), transcription based amplification (Kwoh, D. Y., and Kwoh, T. J. (1990) "Target amplification systems in nucleic acid-based diagnostic approaches," American Biotechnology Laboratory, October, 1990) and strand displacement amplification (SDA) (Walker, G. T., et al. (1992) "Isothermal in vitro amplification of DNA by a restriction enzyme-DNA Polymerase system," Proc. Natl. Acad. Sci., U.S.A. 89: 392-396). Preferably, the DNA sample is subjected to PCR amplification using primer pairs and thermocycling conditions specific to each locus in the set. Reference is made to the Sequence Listing at the end of this specification for details of the primer sequences used in the Examples below, some of which sequences are alternative embodiments of this invention.

Details of the most preferred amplification protocol for each of the most preferred combinations of loci for use in the method of this invention are given in the examples below. Reference is also made to the examples for additional details of the specific procedure relating to each multiplex. The sequences of the locus-specific primers used in the examples include a number of nucleotides which, under the conditions used in the hybridization, are sufficient to hybridize with an allele of the locus to be amplified and to be essentially free from amplification of alleles of other loci. Reference is made to U.S. Pat. No. 5,192,659 to Simons, the teaching of which is incorporated herein by reference for a more detailed description of locus-specific primers.

F. Separation and Detection of DNA Fragments

Once a set of amplified alleles is produced from the multiplex amplification step of the present method, the amplified alleles are evaluated. The evaluation step of this method can be accomplished by any one of a number of different means, the most preferred of which are described

Electrophoresis is preferably used to separate the products of the multiplex amplification reaction, more preferably denaturing polyacrylamide gel electrophoresis (see, e.g., Sambrook, J. et al. (1989) In Molecular Cloning-A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, pp. 13.45–13.57). The most preferred gel preparation and electrophoresis procedures and conditions for use in the evaluating step of the method of this invention are described in Example 1. Separation of DNA fragments in a denaturing polyacrylamide gel occurs based on fragment size.

Once the amplified alleles are separated in a polyacrylamide gel, the alleles and any other DNA in the gel (e.g., DNA markers or an allelic ladder) can then be visualized and analyzed. Visualization of the DNA in the gel can be accomplished using any one of a number of prior art techniques, including silver staining or reporters such as radioisotopes, fluorescers, chemiluminescers and enzymes

in combination with detectable substrates. Silver staining is a preferred method of visualizing the alleles in the gel (see, e.g., Bassam, B. J., et al. (1991) "Fast and sensitive silver staining of DNA in polyacrylamide gels," Anal. Biochem. 196: 80-83). A more preferred method is the use of 5 radioactively-labeled (see, e.g., Hammond et al., (1994)) or fluorescently-labeled (see, e.g., Schumm et al., (1994)) primers for each locus in the multiplexing reaction followed by detection of the labeled products using an autoradiogram or fluorometric detector, respectively. All three references, cited above, which describe prior art methods of visualizing alleles, are incorporated by reference herein.

The alleles present in the DNA sample are preferably determined by comparison to a size standard such as a DNA marker or a locus-specific allelic ladder to determine the 15 fragment of DNA produced from the multiplex amplification alleles present at each locus within the sample. The most preferred size marker for evaluation of a multiplex amplification containing two or more polymorphic STR loci consists of a combination of allelic ladders for each of the loci being evaluated. See, e.g., description of allelic ladders 20 and method of ladder construction in Schumm et al., supra, at p. 178.

The preferred size marker for evaluation of a multiplex amplification containing two or more polymorphic STR loci which are generated using fluorescently-labeled primers for 25 each locus consists of a combination of fluorescently-labeled allelic ladders for the loci being evaluated. Id.

Following the construction of allelic ladders for individual loci, they may be mixed and loaded for gel electrooccurs. Each allelic ladder co-migrates with alleles in the sample from the corresponding locus.

A permanent record of the data can be generated using Automatic Processor Compatible (APC) film (STR systems manual #TMD004, available from Promega Corporation, 35 Madison, Wis.) or with use of a fluorescent detection instrument (STR systems manual #TMD006, also available from Promega Corporation, Madison, Wis.).

G. Preferred Detection Technique: Fluorescent Detection

In one of the most preferred embodiments of the method 40 of this invention, fluorescent detection is used to evaluate the amplified alleles in the mixture produced by the multiplex amplification reaction. Below is a brief summary of how that method of detection preferably is practiced.

With the advent of automated fluorescent imaging, faster 45 detection and analysis of multiplex amplification products can be achieved. For fluorescent analyses, one fluoresceinated primer can be included in the amplification of each locus. Descriptions of the use of two preferred species of fluorescent labeled primers, fluorescein-labeled (FL-) and 50 tetramethyl rhodamine-labeled (TMR-) primers are included in the examples, below. Separation of the amplified fragments produced using such labeled primers is achieved in precisely the same manner as with the silver stain detection method. The resulting gel can be analyzed using a FluorImager™ analyzer (commercially available from Molecular Dynamics, Sunnyvale, Calif.) or FMBIO™ (commercially available from Hitachi Corporation, San Bruno, Calif.), which scans the gel and digitizes the data in a very short time, e.g., three to twenty minutes.

In summary, the method of this invention is most preferably practiced using fluorescent detection at the evaluating step. In this preferred method of detection, one of each pair of primers used in the multiplex amplification reaction has a fluorescent label attached thereto, and as a result, the amplified alleles produced from the amplification reaction are fluorescently labeled. In this most preferred embodiment

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of the invention, the amplified alleles are subsequently separated on a polyacrylamide gel and the separated alleles visualized and analyzed using a fluorescent image analyzer.

Fluorescent detection is preferred over radioactive methods of labeling and detection, because it does not require the use of radioactive materials, and all the regulatory and safety problems which accompany the use of such materials.

Fluorescent detection is also preferred over other nonradioactive methods of detection, such as silver staining, because fluorescent methods of detection generally reveal fewer gel artifacts than staining. The smaller number of gel artifacts are probably due, to a large extent, to the fact that only amplified fragments of DNA with labels attached are detected in fluorescent detection, while every amplified reaction is stained and detected using the silver staining method of detection. Polyacrylamide gels stained with silver stain also have a considerably higher general background due to nonspecific binding of silver stain to the gel itself, reducing the sensitivity with which individual bands of DNA can be detected within the gel. Silver staining and fluorescent methods of detection are compared in two sets of examples, hereinbelow.

H. Kit

The present invention is also directed to kits that utilize the process described above. A basic kit comprises a container having one or more locus-specific primers for each locus. Instructions for use optionally may be included.

Other optional kit components may include an allelic phoresis at the same time as the loading of amplified samples 30 ladder directed to each of the specified loci, a sufficient quantity of enzyme for amplification, amplification buffer to facilitate the amplification, loading solution for preparation of the amplified material for gel electrophoresis, human genomic DNA as a template control, a size marker to insure that materials migrate as anticipated in the gel, and a protocol and manual to educate the user and to limit error in use. The amounts of the various reagents in the kits also can be varied depending upon a number of factors, such as the optimum sensitivity of the process. It is within the scope of this invention to provide test kits for use in manual applications or test kits for use with automated detectors or analyzers.

EXAMPLES

The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the disclosure or protection granted by the patent.

Genomic DNA isolation and quantitation were performed essentially as described by Puers, C., et al. (1993) "Identification of repeat sequence heterogeneity at the polymorphic short tandem repeat locus HUMTH01 [AATG], and reassignment of alleles in population analysis by using a locusspecific allelic ladder," Am. J. Hum. Genet. 53: 953-958. These methods are generally known to those skilled in the art and are preferred, but not required, for application of the invention.

Amplification products were separated by electrophoresis through a 0.4 mm thick 4% denaturing polyacrylamide gel (19:1 ratio of acrylamide to bis-acrylamide) which contained 7M urea (Sambrook et al., (1989)), and which was chemically cross-linked to a glass plate (Kobayashi, Y. (1988) "A method to cast thin sequencing gels," BRL Focus 10: 73–74) in cases involving silver stain analysis. No such crosslinking was employed in cases involving fluorescent analysis. DNA samples were mixed with 2.5 μ l of a loading

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solution (10 MM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured at 95° C. for 2 min., and chilled on ice prior to loading.

Once separated by polyacrylamide gel electrophoresis, 5 the amplified reaction products and DNA size marker controls were detected using silver staining, fluorescent detection, radioactive detection, or a combination of the above detection methods. In some Examples, the reaction products and size markers in the gel were detected by silver staining using a standard method of staining and detection described in the prior art. (See, e.g., Bassam et al., (1991).) Permanent images of the stained gels were obtained by exposure to Automatic Processor Compatible Film (APC Film, Promega Corporation, Cat. No. DQ4411). In other 15 Examples, detection was performed by fluorescent scanning, using a method described in the prior art (Schumm et al., (1994)).

Each example below is an example of the use of the method of this invention, and in some cases, an example of the use of one or more of the primers of this invention to determine simultaneously the alleles present in at least three short tandem repeat loci from one or more DNA samples. Tables 1 and 2 summarize which set of loci was co-amplified in the multiplex amplification reaction described in each Example below. The two tables also indicate which primer pair was used to amplify each such locus in each such multiplex reaction. Table 1 lists all the Examples where fluorescent scanning was used to detect the amplified alleles from the multiplex reactions described therein, while Table 2 lists the Examples where silver staining was used to detect the amplified alleles.

One primer of each primer pair listed on Table 1 was fluorescently labeled prior to being used in the multiplex 35 amplification reaction. In some cases, a different label was used to label primers to different loci, such that the alleles produced using the different primers could be distinguished from one another when scanned by the fluorescent scanner used in the Examples below. Two different fluorescent labels were used in the Examples below, described as "FL" to indicate fluorescein-labeled and "TMR" to indicate tetramethyl rhodamine-labeled in Table 1, below. Table 1 also indicates which primer of each pair of primers used in the multiplex amplification reaction was so labeled in each 45 example (e.g., "FL-2" means the primer with SEQ ID NO:2 was labeled at its 5' end with fluorescein prior to being used in the multiplex amplification reaction).

The same FL and TMR abbreviations are used in the Examples below. However, there the label abbreviation is placed immediately before the SEQ ID NO of the labeled primer used in the amplification reaction described therein (e.g., "FL-SEQ ID NO:2" instead of "FL-2").

In four pairs of Examples below (Examples 2 and 3, 4 and 55, 7 and 8, and 19 and 23), the same set of loci were analyzed using the same set of primers and the same fluorescent labels covalently attached to one of each pair of primers for each STR locus analyzed. However, a different set of primers was labeled in each of the Examples. These pairs of Examples are included herein to demonstrate that the same low background and identical allelic determination results can be obtained from the same set of primers using fluorescent labeling as a method of detection, no matter which of the primers of a primer pair is labeled prior to being used in a 65 multiplex amplification reaction of the method of this invention.

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_	Example	Loci Amplified	Primer Pair: SEQ ID NO:'s	Fluorescent Label(s) Used
	1	D7S820	1, 2	FL-2
		D13S317 D5S818	3, 4 5, 6	FL-4 FL-6
		D3S1539	7, 8	FL-8
	2	D7S820	1, 2	FL-2
		D13S317	3, 4	FL-4
		D5S818 D17S1298	5, 6 9, 10	FL-6 FL-10
	3	D7S820	1, 2	FL-2
		D13S317	3, 4	FL-4
		D5S818 D17S1298	5, 6 9, 10	FL-6 FL-9
	4	D7S820	1, 2	FL-2
		D13S317	3, 4	FL-4
		D5S818 D20S481	5, 6 11, 12	FL-6 FL-12
	5	D7S820	1, 12	FL-2
		D13S317	3, 4	FL-4
		D5S818	5, 6	FL-6
	6	D20S481 D7S820	11, 12 1, 2	FL-11 FL-2
	· ·	D13S317	3, 4	FL-4
		D5S818	5, 6	FL-6
	7	D9S930 D7S820	13, 14 1, 2	FL-14 FL-2
	,	D13S317	3, 4	FL-4
		D5S818	5, 6	FL-6
	8	D10S1239	15, 16	FL-16 FL-2
	0	D7S820 D13S317	1, 2 3, 4	FL-2 FL-4
		D5S818	5, 6	FL-6
	0	D10S1239	15, 16	FL-15
	9	D7S820 D13S317	1, 2 3, 4	FL-2 FL-4
		D5S818	5, 6	FL-6
		D14S118	17, 18	FL-18
	10	D7S820 D13S317	1, 2 3, 4	FL-2 FL-4
		D5S818	5, 6	FL-6
		D14S562	19, 20	FL-19
	11	D7S820 D13S317	1, 2 3, 4	FL-2 FL-4
		D5S818	5, 6	FL-4 FL-6
		D14S548	21, 22	FL-22
	12	D7S820	1, 2	FL-2
		D13S317 D5S818	3, 4 5, 6	FL-4 FL-6
		D16S490	23, 24	FL-23
	13	D7S820	1, 2	FL-2
		D13S317 D5S818	3, 4 5, 6	FL-4 FL-6
		D16S753	25, 26	FL-26
	14	D7S820	1, 2	FL-2
		D13S317 D5S818	3, 4 5, 6	FL-4 FL-6
		D17S1299	27, 28	FL-28
	15	D7S820	1, 2	FL-2
		D13S317	3, 4	FL-4
		D5S818 D16S539	5, 6 29, 30	FL-6 FL-30
	16	D7S820	1, 2	FL-2
		D13S317	3, 4	FL-4
		D5S818 D22S683	5, 6 31, 32	FL-6 FL-32
	17	D7S820	1, 2	FL-2
		D13S317	3, 4	FL-4
		D5S818	5, 6	FL-6
		D16S539 HUMCSF1PO	29, 30 33, 34	FL-30 TMR-33
		HUMTPOX	35, 36	TMR-36
	18	D7S820	1, 2	FL-2
		D13S317 D5S818	3, 4 5, 6	FL-4 FL-6
		D16S539	29, 30	FL-30
		HUMCSF1PO	33, 34	TMR-33
		HUMTPOX HUMTH01	35, 36 37, 38	TMR-36 TMR-38
		HOMINUL	37, 38	1 WIX-30

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TABLE 1-continued

Example	Loci Amplified	Primer Pair: SEQ ID NO:'s	Fluorescent Label(s) Used
19	D7S820	1, 2	FL-2
	D13S317	3, 4	FL-4
	D5S818	5, 6	FL-6
	D16S539	29, 30	FL-30
	HUMCSF1PO	33, 34	TMR-33
	HUMTPOX	35, 36	TMR-36
	HUMTH01	37, 38	TMR-38
	HUMvWFA31	39, 40	TMR-40
20	D7S820	1, 2	FL-2
	D13S317	3, 4	FL-4
	D5S818	5, 6	FL-6
	D16S539	29, 30	FL-30
	HUMF13A01	41, 42	TMR-41
24	HUMFESFPS	43, 44	TMR-43
21	D7S820	1, 2	FL-2
	D13S317	3, 4	FL-4
	D5S818	5, 6	FL-6
	D16S539	29, 30	FL-30
	HUMF13A01	41, 42	TMR-41
	HUMFESFPS	43, 44	TMR-43
22	HUMBFXIII D7S820	45, 46	TMR-45 FL-2
22	D13S317	1, 2 3, 4	FL-2 FL-4
	D5S818	5, 4 5, 6	FL-4 FL-6
	D38818 D168539	29, 30	FL-30
	HUMF13A01	41, 42	TMR-41
	HUMFESFPS	43, 44	TMR-43
	HUMBFXIII	45, 46	TMR-45
	LIPOL	47, 48	TMR-47
23	D7S820	1, 2	TMR-2
20	D13S317	3, 4	TMR-4
	D5S818	5, 6	TMR-6
	D16S539	29, 30	TMR-30
24	D3S1539	7, 49	FL-49
	D19S253	50, 51	FL-50
	D13S317	3, 4	FL-4
	D20S481	52, 53	FL-53
25	D10S1239	15, 54	FL-15
=-	D9S930	55, 14	FL-14
	D4S2368	56, 57	FL-57
	D20S481	52, 53	FL-53

Note that in a few cases, the same set of loci and same set of primer pairs appear in Table 1 and in Table 2. In such cases, the same set of alleles were analyzed using fluorescent detection and silver staining, respectively. Two such cases of duplicate sets are provided herein, Examples 24 and 33, and 45 Examples 25 and 30. These examples clearly illustrate that the same results can be obtained with either method.

TABLE 2

Example	Loci Amplified	Primer Pair: SEQ ID NO:'s
26	D16S539	29, 58
	D7S820	1, 2
	D13S317	3, 4
27	D16S539	29, 30
	D7S820	1, 2
	D13S317	3, 4
	HUMvWFA31	59, 60
28	D10S1239	15, 54
	D9S930	55, 61
	D13S317	31, 4
29	D10S1239	15, 54
	D9S930	55, 61
	D4S2368	56, 57
30	D10S1239	15, 54
	D9S930	55, 14
	D4S2368	56, 57
	D20S481	52, 53

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TABLE 2-continued

5	Example	Loci Amplified	Primer Pair: SEQ ID NO:'s
	31	D3S1539	7, 49
		D19S253	50, 51
		D13S317	3, 4
	32	D3S1539	7, 49
		D19S253	50, 51
		D4S2368	56, 57
		D20S481	52, 53
	33	D3S1539	7, 49
		D19S253	50, 51
		D13S317	3, 4
		D20S481	52, 53
;	34	D10S1239	15, 54
		D9S930	55, 14
		D20S481	52, 53
	35	D10S1239	15, 54
		D4S2368	56, 57
		D20S481	52, 53

Example 1

Flourescent Detection of Multiplex Amplification of Loci D3S1539, D7S820, D13S317, and D5S818

In this example, a DNA template was amplified simultaneously at the individual loci D3S1539, D7S820, and D5S818 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Tag DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Eight amplification primers were used in combination, including $0.25 \,\mu\text{M}$ each D3S1539 primers 1 [SEQ ID NO:7] and 2 [FL-SEQ ID NO:8], $0.325 \,\mu\text{M}$ each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], $0.219 \,\mu\text{M}$ each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], $0.375 \,\mu\text{M}$ each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 45 min. at 40 W and products were visualized by detection of the fluorescent signals using the FluorImagerTM fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 1 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D3S1539, D7S820, D13S317, and D5S818.

Example 2

Fluorescent Detection of Multiplex Amplification of Loci D17S1298. D7S820. D13S317, and D5S818

In this example, a DNA template was amplified simultaneously at the individual loci D17S1298, D7S820, D13S317, and D5S818 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA

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Polymerase/µl. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 5 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Eight amplification primers were used in combination, including 0.25 μ M each D17S1298 primers 1 [SEQ ID NO:9] and 2 [FL-SEQ ID NO:10], 0.325 μ M each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.219 μ M each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], 0.375 μ M each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 45 min. at 40 W and products were visualized by detection of the fluorescent signals using the FluorImagerTM fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 2 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D17S1298, D7S820, D13S317, and D5S818.

Example 3

Fluorescent Detection of Multiplex Amplification of Loci D17S1298, D7S820, D13S317, and D5S818

The loci D17S1298, D7S820, D13S317, and D5S818 were amplified as described in Example 2 except that SEQ 30 ID NO:9 was replaced with FL-SEQ ID NO:9 and FL-SEQ ID NO:10 was replaced with SEQ ID NO:10.

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 45 min. at 40 W and products were visualized by detection of the ³⁵ fluorescent signals using the FluorImagerTM fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 3 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D17S1298, D7S820, D13S317, and D5S818.

Example 4

Fluorescent Detection of Multiplex Amplification of Loci D20S481, D7S820, D13S317, and D5S818

In this example, a DNA template was amplified simultaneously at the individual loci D20S481, D7S820, D13S317, and D5S818 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Eight amplification primers were used in combination, including 0.25 μ M each D20S481 primers 1 [SEQ ID NO:11] and 2 [FL-SEQ ID NO:12], 0.325 μ M each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.219 μ M each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-65 SEQ ID NO:4], 0.375 μ M each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6].

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Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 45 min. at 40 W and products were visualized by detection of the fluorescent signals using the FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 4 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D20S481, D7S820, D13S317, and D5S818.

Example 5

Fluorescent Detection of Multiplex Amplification of Loci D20S481, D7S820. D13S317, and D5S818

The loci D20S481, D7S820, D13S317, and D5S818 were amplified as described in Example 4 except that SEQ ID NO:11 was replaced with FL-SEQ ID NO:11 and FL-SEQ ID NO:12 was replaced with SEQ ID NO:12.

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 45 min. at 40 W and products were visualized by detection of the fluorescent signals using the FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 5 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D20S481, D7S820, D13S317, and D5S818.

Example 6

Fluorescent Detection of Multiplex Amplification of Loci D9S930. D7S820, D13S317, and D5S818

In this example, a DNA template was amplified simultaneously at the individual loci D9S930, D7S820, D13S317, and D5S818 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 30 min.

Eight amplification primers were used in combination, including 0.70 μ M each D9S930 primers 1 [SEQ ID NO:13] and 2 [FL-SEQ ID NO:14], 0.325 μ M each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.22 μ M each D13S317 primers 1 [SEQ ID NO:3) and 2 [FL-SEQ ID NO:4], 0.375 μ M each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 45 min. at 40 W and products were visualized by detection of the fluorescent signals using the FluorImagerTM fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 6 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D9S930, D7S820, D13S317, and D5S818.

Example 7

Fluorescent Detection of Multiplex Amplification of Loci D10S1239, D7S820, D13S317, and D5S818

In this example, a DNA template was amplified simultaneously at the individual loci D10S1239, D7S820,

D13S317, and D5S818 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA 5 Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 10 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Eight amplification primers were used in combination, including 0.75 μ M each D10S1239 primers 1 [SEQ ID NO:15] and 2 [FL-SEQ ID NO:16], 0.325 μ M each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.22 ¹⁵ μ M each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], 0.375 μ M each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 45 min. at 40 W and products were visualized by detection of the fluorescent signals using the FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 7 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D10S1239, D7S820, D13S317, and D55818.

Example 8

Fluorescent Detection of Multiplex Amplification of Loci D10S1239, D7S820, D13S317, and D5S818

The loci D10S1239, D7S820, D13S317, and D5S818 were amplified as described in Example 7 except that SEQ 35 ID NO:15 was replaced with FL-SEQ ID NO:15 and FL-SEQ ID NO:16 was replaced with SEQ ID NO:16.

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 45 min. at 40 W and products were visualized by detection of the fluorescent signals using the FluorImagerTM fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Amplified products were separacrylamide gel electrophoresis on acrylamide gel electrophoresis on acrylam

Reference is made to FIG. 8 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D10S1239, D7S820, D13S317, and D5S818.

Example 9

Fluorescent Detection of Multiplex Amplification of Loci D14S118, D7S820, D13S317, and D5S818

In this example, a DNA template was amplified simultaneously at the individual loci D14S118, D7S820, D13S317, and D5S818 in a single reaction vessel. The PCR amplification was performed in 25 µl of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 µM each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA Polymerase/µl. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Eight amplification primers were used in combination, including $0.50~\mu M$ each D14S118 primers 1 [SEQ ID

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NO:17] and 2 [FL-SEQ ID NO:18], 0.325 μ M each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.22 μ M each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], 0.375 μ M each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 45 min. at 40 W and products were visualized by detection of the fluorescent signals using the FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 9 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D14S118, D7S820, D13S317, and D5S818.

Example 10

Fluorescent Detection of Multiplex Amplification of Loci D14S562. D7S820 D13S317, and D5S818

In this example, a DNA template was amplified simultaneously at the individual loci D14S562, D7S820, D13S317, and D5S818 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 30 min.

Eight amplification primers were used in combination, including 0.50 μ M each D14S562 primers 1 [FL-SEQ ID NO:19] and 2 [SEQ ID NO:20], 0.325 μ M each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.22 μ M each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], 0.375 μ M each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEO ID NO:6].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 45 min. at 40 W and products were visualized by detection of the fluorescent signals using the FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 10 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D14S562, D7S820, D13S317, and D5S818.

Example 11

Fluorescent Detection of Multiplex Amplification of Loci D14S548, D7S820, D13S317, and D5S818

In this example, a DNA template was amplified simultaneously at the individual loci D14S548, D7S820, D13S317, and D5S818 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

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Eight amplification primers were used in combination, including 0.50 µM each D14S548 primers 1 [SEQ ID NO:21] and 2 [FL-SEQ ID NO:22], 0.325 μ M each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.22 μM each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL- 5 SEQ ID NO:4], 0.375 μM each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 45 min. at 40 W and products were visualized by detection of the 10 fluorescent signals using the FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 11 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D14S548, D7S820, D13S317, and D5S818.

Example 12

Fluorescent Detection of Multiplex Amplification of Loci D16S490, D7S820, D13S317, and D5S818

In this example, a DNA template was amplified simultaneously at the individual loci D16S490, D7S820, D13S317, and D5S818 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA Polymerase/µl. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Eight amplification primers were used in combination, including 0.50 µM each D16S490 primers 1 [FL-SEQ ID NO:23] and 2 [SEQ ID NO:24], 0.325 µM each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.22 uM each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], 0.375 μ M each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 45 min. at 40 W and products were visualized by detection of the 45 fluorescent signals using the FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 12 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D16S490, 50 D7S820, D13S317, and D5S818.

Example 13

Fluorescent Detection of Multiplex Amplification of Loci D16S753, D7S820, D13S317, and D5S818

In this example, a DNA template was amplified simultaneously at the individual loci D16S753, D7S820, D13S317, and D5S818 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 µM each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA Polymerase/µl. A Thermal Cycler 480 (Perkin Elmer, Foster protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by

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20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Eight amplification primers were used in combination, including 0.50 μM each D16S753 primers 1 [SEQ ID NO:25] and 2 [FL-SEQ ID NO:26], 0.325 μM each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.22 μM each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], 0.375 μ M each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 45 min. at 40 W and products were visualized by detection of the fluorescent signals using the FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 13 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D16S753, D7S820, D13S317, and D5S818.

Example 14

Fluorescent Detection of Multiplex Amplification of Loci D17S1299. D7S820. D13S317, and D5S818

In this example, a DNA template was amplified simultaneously at the individual loci D17S1299, D7S820, D13S317, and D5S818 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA Polymerase/μl. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Eight amplification primers were used in combination, including 0.50 µM each D17S1299 primers 1 [SEQ ID NO:27] and 2 [FL-SEQ ID NO:28], 0.325 μM each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.22 μM each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], 0.375 μ M each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 45 min. at 40 W and products were visualized by detection of the fluorescent signals using the FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 14 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D17S1299, D7S820, D13S317, and D5S818.

Example 15

Fluorescent Detection of Multiplex Amplification of Loci D16S539, D7S820, D13S317, and D5S818

In this example, a DNA template was amplified simultaneously at the individual loci D16S539, D7S820, D13S317, and D5S818 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton City, Calif.) was employed with the following amplification 65 X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA Polymerase/μl. A Thermal Cycler 480 (Perkin Elmer, Foster

City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Eight amplification primers were used in combination, including 0.60 µM each D16S539 primers 1 [SEQ ID NO:29] and 2 [FL-SEQ ID NO:30], 0.325 μ M each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.22 μM each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], $0.50 \,\mu\text{M}$ each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 50 min. at 40 W and products were visualized by detection of the fluorescent signals using the FluorImagerTM fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 15 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA $_{20}$ samples simultaneously co-amplified for the loci D16S539, D7S820, D13S317, and D5S818.

Example 16

Fluorescent Detection of Multiplex Amplification of Loci D22S683, D7S820, D13S317, and D5S818

In this example, a DNA template was amplified simultaneously at the individual loci D22S683, D7S820, D13S317, and D5S818 in a single reaction vessel. The PCR amplifi- 30 cation was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA Polymerase/µl. A Thermal Cycler 480 (Perkin Elmer, Foster 35 City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Eight amplification primers were used in combination, including 0.50 µM each D22S683 primers 1 [SEQ ID NO:31] and 2 [FL-SEQ ID NO:32], 0.325 μ M each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.22 μM each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], 0.375 μM each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 55 min. at 40 W and products were visualized by detection of the fluorescent signals using the FluorImager $^{\text{TM}}$ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 16 which displays the amplisamples simultaneously co-amplified for the loci D22S683, D7S820, D13S317, and D5S818.

Example 17

Fluorescent Detection of Multiplex Amplification of Loci D16S539, D7S820, D13S317, D5S818, **HUMCSF1PO** and **HUMTPOX**

In this example, a DNA template was amplified simultaneously at the individual loci D16S539, D7S820, D13S317, 65 D5S818, HUMCSF1PO and HUMTPOX in a single reaction vessel. The PCR amplification was performed in 25 μ l

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of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μM each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.06 U Taq DNA Polymerase/μl. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° 10 C. for 30 min.

Twelve amplification primers were used in combination, including 0.65 μ M each D16S539 primers 1 [SEQ ID NO:29] and 2 [FL-SEQ ID NO:30], 0.325 μM each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.22 uM each D13S317 primers 1 [SEQ. ID NO:3] and 2 [FL-SEQ ID NO:4], 0.55 μ M each D5S818 primers 1 [SEQ ID NO:5] and $\tilde{2}$ [FL-SEQ ID NO:6], 0.40 μM each HUMCSF1PO primers 1 [TMR-SEQ ID NO:33] and 2 [SEQ ID NO:34], 0.40 $\mu \dot{M}$ each HUMTPOX primers 1 [SEQ ID NO:35] and 2 [TMR-SEQ ID NO:36].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 50 min. at 40 W and products were visualized by detection of the fluorescent signals using the FMBIOFluorImager™ (Hitachi Software Engineering, San Bruno, Calif.).

Reference is made to FIG. 17 which displays the amplified fragments of each locus in separate 505 nm and 625 nm scans of the same gel revealing fluorescein-labeled and tetramethyl-rhodamine labeled material, respectively. Lanes 1 to 4 contain DNA samples simultaneously co-amplified for the loci D16S539, D7S820, D13S317, D5S818, HUMCSF1PO ("CSF1PO") and HUMTPOX ("TPOX").

Example 18

Fluorescent Detection of Multiplex Amplification of Loci D16S539. D7S820, D13S317, D5S818. HUMCSF1PO, HUMTPOX and HUMTH01

In this example, a DNA template was amplified simultaneously at the individual loci D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, HUMTPOX and HUMTH01 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 µM each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0. 07 U Taq DNA Polymerase/µl. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Fourteen amplification primers were used in combination, fied fragments of each locus. Lanes 1 to 5 contain DNA $_{55}$ including 0.75 μ M each D16S539 primers 1 [SEQ ID NO:29] and 2 [FL-SEQ ID NO:30], 0.40 μM each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.30 μM each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], 0.60 μ M each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6], 0.30 μ M each HUMCSF1PO primers 1 [TMR-SEQ ID NO:33] and 2 [SEQ ID NO:34], 0.40 μ M each HUMTPOX primers 1 [SEQ ID NO:35] and 2 [TMR-SEQ ID NO:35], 0.40 μ M each HUMTH01 primers 1 [SEQ ID NO:37] and 2 [TMR-SEQ ID NO:38].

> Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 50 min. at

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40 W and products were visualized by detection of the fluorescent signals using the FMBIOFluorImager™ (Hitachi Software Engineering, San Bruno, Calif.).

Reference is made to FIG. 18 which displays the amplified fragments of each locusin separate 505 nm and 625 nm 5 scans of the same gel revealing fluorescein-labeled and tetramethyl-rhodamine labeled material, respectively. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D16S539, D7S820, D13S317, D5S818, HUMTH01 ("TH01").

Example 19

Fluorescent Detection of Multiplex Amplification of Loci D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, HUMTPOX, HUMTH01 and HUMvWFA31

In this example, a DNA template was amplified simultaneously at the individual loci D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, HUMTPOX, HUMTH01 and HUMvWFA31 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, ²⁵ dGTP and dTTP) using 5 ng template, and 0.08 U Taq DNA Polymerase/µl. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Sixteen amplification primers were used in combination, including 0.75 µM each D16S539 primers 1 [SEQ ID NO:29] and 2 [FL-SEQ ID NO:30], 0.40 µM each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.30 μM each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], 0.60 µM each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6], 0.30 μ M each HUMCSF1PO primers 1 [TMR-SEQ ID NO:33] and 2 [SEQ ID NO:34], 0.40 μ M each HUMTPOX primers 1 [SEQ ID NO:35] and 2 [TMR-SEQ ID NO:36], 0.40 μ M each HUMTH01 primers 1 [SEQ ID NO:37] and 2 [TMR-SEQ ID NO:38], 0.40 µM each HUMvWFA31 primers 1 [SEQ ID NO:39] and 2 [TMR-SEQ ID NO:40].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 50 min. at 40 W and products were visualized by detection of the fluorescent signals using the FMBIOFluorImager™ (Hitachi 50 Software Engineering, San Bruno, Calif.).

Reference is made to FIG. 19 which displays the amplified fragments of each locus in separate 505 nm and 625 nm scans of the same gel revealing fluorescein-labeled and tetramethyl-rhodamine labeled material, respectively. Lanes 55 1 to 5 contain DNA samples simultaneously co-amplified for the loci D16S539, D7S820, D13S317, D5S818, HUMCSF1PO ("CSF1PO"), HUMTPOX ("TPOX"), HUMTH01 ("TH01") and HUMvWFA31 ("vWA").

Example 20

Fluorescent Detection of Multiplex Amplification of Loci D16S539, D7S820, D13S317, D5S818, HUMF13A01 and HUMFESFPS

In this example, a DNA template was amplified simultaneously at the individual loci D16S539, D7S820, D13S317, **30**

D5S818, HUMF13A01 and HUMFESFPS in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.06 U Taq DNA Polymerase/μl. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for HUMCSF1PO ("CSF1PO"), HUMTPOX ("TPOX") and 10 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

> Twelve amplification primers were used in combination, including 0.75 μ M each D16S539 primers 1 [SEQ ID 15 NO:29] and 2 [FL-SEQ ID NO:30], 0.40 μM each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.30 μM each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], 0.60 μ M each D5S818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6], 0.10 μ M each HUMF13A01 primers 1 [TMR-SEQ ID NO:41] and 2 [SEQ ID NO:42], 1.0 μM each HUMFESFPS primers 1 [TMR-SEQ ID NO:43] and 2 [SEQ ID NO:44].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 50 min. at 40 W and products were visualized by detection of the fluorescent signals using the FMBIOFluorImager™ (Hitachi Software Engineering, San Bruno, Calif.).

Reference is made to FIG. 20 which displays the amplified fragments of each locus in separate 505 nm and 625 nm scans of the same gel revealing fluorescein-labeled and tetramethyl-rhodamine labeled material, respectively. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D16S539, D7S820, D13S317, D5S818, HUMF13A10 ("F13A01") and HUMFESFPS ("FESFPS").

Example 21

Fluorescent Detection of Multiplex Amplification of Loci D16S539, D7S820, D13S317, D5S818, HUMF13A01. HUMFESFPS and HUMBFXIII

In this example, a DNA template was amplified simultaneously at the individual loci D16S539, D7S820, D13S317, D5S818, HUMF13A01, HUMFESFPS and HUMBFXIII in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 µM each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.07 U Taq DNA Polymerase/µl. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Fourteen amplification primers were used in combination, including 0.75 µM each D16S539 primers 1 [SEQ ID NO:29] and 2 [FL-SEQ ID NO:30], 0.40 μM each D7S820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.30 μM each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], $0.60 \mu M$ each D5S818 primers 1 [SEQ ID NO:5] and $\tilde{2}$ [FL-SEQ ID NO:6], 0.10 μ M each HUMF13A01 primers 1 [TMR-SEQ ID NO:41] and 2 [SEQ ID NO:42], 1.0 μM each HUMFESFPS primers 1 [TMR-SEQ ID NO:43] and 2 [SEQ ID NO:44], 0.50 μ M each HUMBFXIII primers 1 [TMR-SEQ ID NO:45] and 2 [SEQ ID NO:46].

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Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 50 min. at 40 W and products were visualized by detection of the fluorescent signals using the FNBIOFluorImager™ (Hitachi Software Engineering, San Bruno, Calif.).

Reference is made to FIG. 21 which displays the amplified fragments of each locus in separate 505 nm and 625 nm scans of the same gel revealing fluorescein-labeled and tetramethyl-rhodamine labeled material, respectively. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D168539, D78820, D13S317, D5S818, HUMF13A01 ("F13A01"), HUMFESFPS ("FESFPS") and HUMBFXIII ("F13B").

Example 22

Fluorescent Detection of Multiplex Amplification of Loci D16S539, D7S820, D13S317, D5S818, HUMF13A10, HUMFESFPS, HUMBFXIII and HUMLIPOL

In this example, a DNA template was amplified simultaneously at the individual loci D16S539, D7S820, D13S317, D5S818, HUMF13A10, HUMFESFPS, HUMBFXIII and HUMLIPOL in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, 25 dGTP and dTTP) using 5 ng template, and 0.08 U Taq DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., 60° C. for 1 min., 60° C. for 1 min., 50° C. for 1 min., 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 30 min.

Sixteen amplification primers were used in combination, including 0.75 μ M each D168539 primers 1 [SEQ ID NO:29] and 2 [FL-SEQ ID NO:30], 0.40 μ M each D78820 primers 1 [SEQ ID NO:1] and 2 [FL-SEQ ID NO:2], 0.30 μ M each D138317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], 0.60 μ M each D58818 primers 1 [SEQ ID NO:5] and 2 [FL-SEQ ID NO:6], 0.10 μ M each HUMF13A01 primers 1 [TMR-SEQ ID NO:41] and 2 [SEQ ID NO:42], 1.0 μ M each HUMFESFPS primers 1 [TMR-SEQ ID NO:43] and 2 [SEQ ID NO:44], 0.50 μ M each HUMBFXIII primers 1 [TMR-SEQ ID NO:45] and 2 [SEQ ID NO:46], 0.20 μ M each HUMLIPOL primers 1 [TMR-SEQ ID NO:47] and 2 [SEQ ID NO:48].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 50 min. at 40 W and products were visualized by detection of the fluorescent signals using the FMBIOFluorImager™ (Hitachi Software Engineering, San Bruno, Calif.).

Reference is made to FIG. 22 which displays the amplified fragments of each locus in separate 505 nm and 625 nm scans of the same gel revealing fluorescein-labeled and tetramethyl-rhodamine labeled material, respectively. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D16S539, D7S820, D13S317, D5S818, HUMF13A01 ("HUMF13A01"), HUMFESFPS ("FESFPS"), HUMBFXIII ("F13B") and HUMLIPOL ("LPL").

Example 23

Fluorescent Detection of Multiplex Amplification of Loci D16S539. D7S820. D13S317, D5S818, HUMCSF1PO, HUMTPOX, HUMTH01 and HUMvWFA31

In this example, a DNA template was amplified simultaneously at the individual loci D16S539, D7S820, D13S317,

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D5S818, HUMCSF1PO, HUMTPOX, HUMTH01 and HUMvWFA31 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.08 U Tag DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., followed by 1 cycle of 60° C. for 30 min.

Sixteen amplification primers were used in combination, including 0.75 μM each D16S539 primers 1 [SEQ ID NO:29] and 2 [TMR-SEQ ID NO:30], 0.40 μM each D7S820 primers 1 [SEQ ID NO:1] and 2 [TMR-SEQ ID NO:2], 0.30 μM each D13S317 primers 1 [SEQ ID NO:3] and 2 [TMR-SEQ ID NO:4], 0.60 μM each D5S818 primers 1 [SEQ ID NO:5] and 2 [TMR-SEQ ID NO:6], 0.40 μM each HUMCSF1PO primers 1 [FL-SEQ ID NO:33] and 2 [SEQ ID NO:35] and 2 [FL-SEQ ID NO:36], 0.20 μM each HUMTHO1 primers 1 [SEQ ID NO:37] and 2 [FL-SEQ ID NO:38], 0.55 μM each HUMVWFA31 primers 1 [SEQ ID NO:38] and 2 [FL-SEQ ID NO:40].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 50 min. at 40 W and products were visualized by detection of the fluorescent signals using the FMBIOFluorImagerTM (Hitachi Software Engineering, San Bruno, Calif.).

Reference is made to FIG. 23 which displays the amplified fragments of each locus in separate 505 nm and 625 nm scans of the same gel revealing fluorescein-labeled and tetramethyl-rhodamine labeled material, respectively. Lanes 1 to 3 contain DNA samples simultaneously co-amplified for the loci D16S539, D7S820, D13S317, D5S818, HUMCSF1PO ("CSF1PO"), HUMTPOX ("TPOX"), HUMTH01 ("TH01") and HUMvWFA31 ("vWA").

Example 24

Fluorescent Detection of Multiplex Amplification of Loci D3S1539, D19S253, D13S317, and D2OS481

In this example, a DNA template was amplified simultaneously at the individual loci D3S1539, D19S253, D13S317 and D20S481 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 30 min.

Eight amplification primers were used in combination, including $0.75 \,\mu\text{M}$ each D3S1539 primers 1 [SEQ ID NO:7] and 2 [FL-SEQ ID NO:49], $0.75 \,\mu\text{M}$ each D19S253 primers 1 [FL-SEQ ID NO:50] and 2 [SEQ ID NO:51], $0.50 \,\mu\text{M}$ each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], $0.50 \,\mu\text{M}$ each D20S481 primers 1 [SEQ ID NO:52] and 2 [FL-SEQ ID NO:53].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 50 min. at 40 W and products were visualized by detection of the

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fluorescent signals using the FluorImager™ fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. 24 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D3S1539, 5 D19S253, D13S317 and D20S481.

Example 25

Fluorescent Detection of Multiplex Amplification of Loci D10S1239, D9S930, D4S2368, and D20S481

In this example, a DNA template was amplified simultaneously at the individual loci D10S1239, D9S930, D4S2368 and D20S481 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Eight amplification primers were used in combination, including 0.30 μM each D10S1239 primers 1 [FL-SEQ ID NO:15] and 2 [SEQ ID NO:54], 0.40 μM each D9S930 primers 1 [SEQ ID NO:55] and 2 [FL-SEQ ID NO:14], 0.50 μM each D4S2368 primers 1 [SEQ ID NO:56] and 2 30 [FL-SEQ ID NO:57], 0.50 μM each D2OS481 primers 1 [SEQ ID NO:52] and 2 [FL-SEQ ID NO:53].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 50 min. at 40 W and products were visualized by detection of the 35 fluorescent signals using the FluorImagerTM fluorescent scanner (Molecular Dynamics, Sunnyvale, Calif.).

Reference is made to FIG. **25** which displays the amplified fragments of each locus. Lanes **1** to **5** contain DNA samples simultaneously co-amplified for the loci D10S1239, ⁴⁰ D9S930, D4S2368 and D20S481.

Example 26

Silver Detection of Multiplex Amplification of Loci D16S539, D7S820 and D13S317

In this example, a DNA template was amplified simultaneously at the individual loci, D16S539, D7S820, and D13S317 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 to 25 ng template, and 0.03 U Taq DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., 60° C. for 1 min., 50° C. for 1 min., 70° C. for 1.5 min, followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min, followed by 1 cycle of 60° C. for 30 min.

Six amplification primers were used in combination, including 0.5 μ M each D16S539 primers 1 [SEQ ID NO:29] and 2 [SEQ ID NO:58], 0.5 μ M each D7S820 primers 1 [SEQ ID NO:1] and 2 [SEQ ID NO:2], 0.5 μ M each D13S317 primers 1 [SEQ ID NO:3] and 2 [SEQ ID NO:4].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 50 min. at

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40 W and products were visualized by silver stain analysis according to the protocol of Bassam et al.(1991).

Reference is made to FIG. 26 which displays the amplified fragments of each locus. Lanes 1 to 4 contain DNA samples simultaneously co-amplified for the loci D16S539, D7S820, and D13S317 and lane 5 displays a sample without DNA template subjected to the same procedures, i.e., a negative control.

Example 27

Silver Detection of Multiplex Amplification of Loci D16S539, D7S820, D13S317 and HUMvWFA31

In this example, a DNA template was amplified simultaneously at the individual loci, D168539, D78820, D138317 and HUMvWFA31 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1. 5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min, followed by 1 cycle of 60° C. for 30 min.

Eight amplification primers were used in combination, including $0.3 \,\mu\text{M}$ each D16S539 primers 1 [SEQ ID NO:29) and 2 [SEQ ID NO:30], $0.3 \,\mu\text{M}$ each D7S820 primers 1 [SEQ ID NO:13 and 2 [SEQ ID NO:2], $0.5 \,\mu\text{M}$ each D13S317 primers 1 [SEQ ID NO:3] and 2 (SEQ ID NO:4], $0.5 \,\mu\text{M}$ each HUMvWFA31 primers 1 [SEQ ID NO:59] and 2 [SEQ ID NO:60].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 32 cm gel for 50 min. at 40 W and products were visualized by silver stain analysis according to the protocol of Bassam et al.(1991).

Reference is made to FIG. 27 which displays the amplified fragments of each locus. Lanes 1 to 3 contain DNA samples simultaneously co-amplified for the loci D16S539, D7S820, D13S317 and HUMvWFA31 ("vWA").

Example 28

Silver Detection of Multiplex Amplification of Loci D10S1239, D9S930, and D13S317

In this example, a DNA template was amplified simultaneously at the individual loci, D10S1239, D9S930, and D13S317 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using undetermined ng template, and 0.03 U Tag DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 minutes.

Six amplification primers were used in combination, including 1.0 μ M each D10S1239 primers 1 [SEQ ID NO:15] and 2 [SEQ ID NO:54], 0.3 μ M each D9S930 primers 1 [SEQ ID NO:55] and 2 [SEQ ID NO:61], 0.5 μ M each D13S317 primers 1 [SEQ ID NO:3] and 2 [SEQ ID NO:4].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 40 cm gel for 60 min. at

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60 W and products were visualized by silver stain analysis according to the protocol of Bassam et al.(1991).

Reference is made to FIG. 28 which displays the amplified fragments of each locus. Lanes 1 to 3 contain DNA samples simultaneously co-amplified for the loci ⁵ D10S1239, D9S930, and D13S317.

Example 29

Silver Detection of Multiplex Amplification of Loci D10S1239, D9S930, and D4S2368

In this example, a DNA template was amplified simultaneously at the individual loci, D10S1239, D9S930, and D4S2368 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using undetermined ng template, and 0.03 U Taq DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 30 min. 25

Six amplification primers were used in combination, including 1.0 μ M each D10S1239 primers 1 [SEQ ID NO:15] and 2 [SEQ ID NO:54], 0.3 μ M each D9S930 primers 1 [SEQ ID NO:55] and 2 [SEQ ID NO:61], 0.15 μ M each D4S2368 primers 1 (SEQ ID NO:56] and 2 [SEQ ID NO:57].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 40 cm gel for 60 min. at 60 W and products were visualized by silver stain analysis according to the protocol of Bassam et al.(1991).

Reference is made to FIG. 29 which displays the amplified fragments of each locus. Lanes 1 to 6 contain DNA samples simultaneously co-amplified for the loci D10S1239, D9S930, and D4S2368.

Example 30

Silver Detection of Multiplex Amplification of Loci D10S1239, D9S930, D4S2368 and D20S481

In this example, a DNA template was amplified simultaneously at the individual loci, D10S1239, D9S930, D4S2368 and D20S481 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.55 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using undetermined ng template, and 0.04 U Taq DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., 60° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Eight amplification primers were used in combination, including 1.0 μ M each D10S1239 primers 1 [SEQ ID NO:15] and 2 [SEQ ID NO:54], 4.0 μ M each D9S930 primers 1 [SEQ ID NO:55] and 2 [SEQ ID NO:14], 0.2 μ M each D4S2368 primers 1 [SEQ ID NO:56] and 2 [SEQ ID NO:57] and 0.2 μ M each D20S481 primers 1 [SEQ ID NO:52] and 2 [SEQ ID NO:53].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 40 cm gel for 67 min. at

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60 W and products were visualized by silver stain analysis according to the protocol of Bassam et al.(1991).

Reference is made to FIG. 30 which displays the amplified fragments of each locus. Lanes 1 to 4 contain DNA samples simultaneously co-amplified for the loci D10S1239, D9S930, D4S2368 and D2OS481.

Example 31

Silver Detection of Multiplex Amplification of Loci D3S1539, D19S253 and D13S317

In this example, a DNA template was amplified simultaneously at the individual loci, D3S1539, D19S253 and D13S317 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5.0 ng template, and 0.03 U Tag DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min.

Six amplification primers were used in combination, including 1.0 μ M each D3S1539 primers 1 [SEQ ID NO:7] and 2 [SEQ ID NO:49], 1.0 μ M each D19S253 primers 1 [SEQ ID NO:50] and 2 [SEQ ID NO:51], 0.5 μ M each D13S317 primers 1 [SEQ ID NO:3] and 2 [SEQ ID NO:4].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 40 cm gel for 65 min. at 60 W and products were visualized by silver stain analysis according to the protocol of Bassam et al.(1991).

Reference is made to FIG. 31 which displays the amplified fragments of each locus. Lanes 1 to 4 contain DNA samples simultaneously co-amplified for the loci D3S1539, D19S253 and D13S317 and lane 5 displays a sample without DNA template subjected to the same procedures, i.e., a negative control.

Example 32

Silver Detection of Multiplex Amplification of Loci D3S1539, D19S253, D4S2368 and D20S481

In this example, a DNA template was amplified simultaneously at the individual loci, D3S1539, D19S253, D4S2368 and D20S481 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 5 ng template, and 0.04 U Taq DNA Polymerase/ μ l. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min.

Eight amplification primers were used in combination, including 1.0 μM each D3S1539 primers 1 [SEQ ID NO:7] and 2 [SEQ ID NO:49], 0.5 μM each D19S253 primers 1 [SEQ ID NO:50] and 2 [SEQ ID NO:51], 0.1 μM each D4S2368 primers 1 [SEQ ID NO:56] and 2 [SEQ ID NO:57]], 0.1 μM each D20S481 primers 1 [SEQ ID NO:52] and 2 [SEQ ID NO:53].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 40 cm gel for 65 min. at

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60 W and products were visualized by silver stain analysis according to the protocol of Bassam et al.(1991).

Reference is made to FIG. 32 which displays the amplified fragments of each locus. Lanes 1 to 4 contain DNA 5 samples simultaneously co-amplified for the loci D3S1539, D19S253, D4S2368 and D20S481 and lane 5 displays a sample without DNA template subjected to the same procedures, i.e., a negative control.

Example 33

Silver Detection of Multiplex Amplification of Loci D3S1539, D19S253, D13S317 and D20S481

In this example, a DNA template was amplified simultaneously at the individual loci, D3S1539, D19S253, D13S317 and D20S481 in a single reaction vessel. The PCR $\ ^{20}$ amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 0.5 to 250 ng template, and 0.04 U Taq DNA Polymerase/µl. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 30 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Eight amplification primers were used in combination, including 0.5 μ M each D3S1539 primers 1 [SEQ ID NO:7] and 2 [SEQ ID NO:49], 0.5 μ M each D19S253 primers 1 35 [SEQ ID NO:50] and 2 (SEQ ID NO:51], 0.5 μ M each D13S317 primers 1 (SEQ ID NO:3] and 2 [SEQ ID NO:4], $0.2 \mu M$ each D20S481 primers 1 [SEQ ID NO:52] and 2 [SEQ ID NO:53].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 40 cm gel for 68 min. at 60 W and products were visualized by silver stain analysis according to the protocol of Bassam et al.(1991).

Reference is made to FIG. 33 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D3S1539, D19S253, D13S317 and D20S481 and lane 6 displays a procedures, i.e., a negative control.

Example 34

Silver Detection of Multiplex Amplification of Loci D10S1239, D9S930 and D20S481

In this example, a DNA template was amplified simultaneously at the individual loci, D10S1239, D9S930 and D20S481 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 μ M each of dATP, dCTP, dGTP and 65 D4S2368 and D20S481 and lane 6 displays a sample withdTTP) using 0.5 to 250 ng template, and 0.03 U Taq DNA Polymerase/μl. A Thermal Cycler 480 (Perkin Elmer, Foster

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City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Six amplification primers were used in combination, including 1.0 µM each D10S1239 primers 1 [SEQ ID NO:15] and 2 [SEQ ID NO:54], 4.0 µM each D9S930 primers 1 [SEQ ID NO:55] and 2 [SEQ ID NO:14], and 0.2 μM each D20S481 primers 1 [SEQ ID NO:52] and 2 [SEQ ID NO:53].

Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 40 cm gel for 68 min. at 60 W and products were visualized by silver stain analysis according to the protocol of Bassam et al.(1991).

Reference is made to FIG. 34 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D10S1239, D9S930 and D20S481 and lane 6 displays a sample without DNA template subjected to the same procedures, i.e., a negative control.

Example 35

Silver Detection of Multiplex Amplification of Loci D10S1239, D4S2368 and D20S481

In this example, a DNA template was amplified simultaneously at the individual loci, D10S1239, D4S2368 and D20S481 in a single reaction vessel. The PCR amplification was performed in 25 μ l of 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ and 200 µM each of dATP, dCTP, dGTP and dTTP) using 0.5 to 250 ng template, and 0.03 U Tag DNA Polymerase/µl. A Thermal Cycler 480 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 2 min., then 10 cycles of 94° C. for 1 45 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 60° C. for 1 min., 70° C. for 1.5 min., followed by 1 cycle of 60° C. for 30 min.

Six amplification primers were used in combination, sample without DNA template subjected to the same 50 including 1.0 µM each D10S1239 primers 1 [SEO ID NO:15] and 2 [SEQ ID NO:54], 0.2 μ M each D4S2368 primers 1 [SEQ ID NO:56] and 2 [SEQ ID NO:57], and 0.2 μM each D20S481 primers 1 [SEQ ID NO:52] and 2 [SEQ ₅₅ ID NO:53].

> Amplified products were separated by denaturing polyacrylamide gel electrophoresis on a 40 cm gel for 68 min. at 60 W and products were visualized by silver stain analysis according to the protocol of Bassam et al.(1991).

> Reference is made to FIG. 35 which displays the amplified fragments of each locus. Lanes 1 to 5 contain DNA samples simultaneously co-amplified for the loci D10S1239, out DNA template subjected to the same procedures, i.e., a negative control.

39 40

SEQUENCE LISTING	
(1) GENERAL INFORMATION:	
(i i i) NUMBER OF SEQUENCES: 61	
(2) INFORMATION FOR SEQ ID NO:1:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear	
(i i) MOLECULE TYPE: Human Genomic DNA	
(i i i) HYPOTHETICAL: no	
(vii) POSITION IN GENOME: (B) MAP POSITION: D7S820	
$(\ x\ i\)$ SEQUENCE DESCRIPTION: SEQ ID NO:1:	
GAACACTTGT CATAGTTTAG AACG	2 4
(2) INFORMATION FOR SEQ ID NO:2:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 23 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear	
(viii) POSITION IN GENOME: (B) MAP POSITION: D78820	
$(\ x\ i\)$ SEQUENCE DESCRIPTION: SEQ ID NO:2:	
CTGAGGTATC AAAAACTCAG AGG	2 3
(2) INFORMATION FOR SEQ ID NO:3:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear	
(viii) POSITION IN GENOME: (B) MAP POSITION: D13S317	
(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:	
ACAGAAGTCT GGGATGTGGA	2 0
(2) INFORMATION FOR SEQ ID NO:4:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear	
(viii) POSITION IN GENOME: (B) MAP POSITION: D13S317	
(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:	
GCCCAAAAG ACAGACAGAA	2 0
(2) INFORMATION FOR SEQ ID NO:5:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 20	

42 41 -continued (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (v i i i) POSITION IN GENOME: (B) MAP POSITION: D5S818 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:5: GGGTGATTTT CCTCTTTGGT 2 0 (2) INFORMATION FOR SEQ ID NO:6: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: $20\,$ (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($\mathbf{v}\ \mathbf{i}\ \mathbf{i}\ \mathbf{i}$) POSITION IN GENOME: (B) MAP POSITION: D5S818 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:6: TGATTCCAAT CATAGCCACA 2 0 $(\ 2\)$ INFORMATION FOR SEQ ID NO:7: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 26 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (viii) POSITION IN GENOME: (B) MAP POSITION: D3S1539 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:7: TCTCTTTCCA TTACTCTCTC CATAGC 2 6 (2) INFORMATION FOR SEQ ID NO:8: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 22 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (v i i i) POSITION IN GENOME: (B) MAP POSITION: D3S1539 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:8: AGTGCTGTTT TAGCTTCCAG GA 2 2 (2) INFORMATION FOR SEQ ID NO:9: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($\mathbf{v} \;\; \mathbf{i} \;\; \mathbf{i} \;\; \mathbf{i} \;\;)$ POSITION IN GENOME: (B) MAP POSITION: D17S1298 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:9: GTAGGTCTTT TGGTTGCCAG TATG 2 4 (2) INFORMATION FOR SEQ ID NO:10: (i) SEOUENCE CHARACTERISTICS: (A) LENGTH: 24

43 44 -continued (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (v i i i) POSITION IN GENOME: (B) MAP POSITION: D17S1298 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:10: TGTCAGTAAA CCTGTGACCT GAGT 2.4 (2) INFORMATION FOR SEQ ID NO:11: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 26 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($\mathbf{v}\ \mathbf{i}\ \mathbf{i}\ \mathbf{i}$) POSITION IN GENOME: (B) MAP POSITION: D20S481 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:11: AATGGTGAGA AATGGGTTAT GAGTGC $(\ 2\)$ INFORMATION FOR SEQ ID NO:12: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 25 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (viii) POSITION IN GENOME: (B) MAP POSITION: D20S481 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:12: TTTCCGGCTT TGTGTCATAA AACAG 2.5 $(\ 2\)$ INFORMATION FOR SEQ ID NO:13: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear $(\ v\ i\ i\ i\)$ Position in Genome: (B) MAP POSITION: D9S930 (\mathbf{x} i) SEQUENCE DESCRIPTION: SEQ ID NO:13: TGGACAACAG AGTGAGATGC 2 0 (2) INFORMATION FOR SEQ ID NO:14: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 23 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (viii) POSITION IN GENOME: (B) MAP POSITION: D9S930 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:14: GCTATGGGAA TTACAAGCAG GAA 2 3 (2) INFORMATION FOR SEQ ID NO:15: (i) SEOUENCE CHARACTERISTICS: (A) LENGTH: 26

45 46 -continued (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (v i i i) POSITION IN GENOME: (B) MAP POSITION: D10S1239 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:15: CTTTGAAATG GACCCCTAGC TAATGT 2 6 (2) INFORMATION FOR SEQ ID NO:16: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: $21\,$ (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($\mathbf{v}\ \mathbf{i}\ \mathbf{i}\ \mathbf{i}$) POSITION IN GENOME: ($\,B\,$) MAP POSITION: D10S1239 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:16: CACCCTGTCC CCAGCTATCT G $(\ 2\)$ INFORMATION FOR SEQ ID NO:17: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 19 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (viii) POSITION IN GENOME: (B) MAP POSITION: D14S118 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:17: CAGCTTGGGC AACATAGGG 19 (2) INFORMATION FOR SEQ ID NO:18: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear $(\ v\ i\ i\ i\)$ Position in Genome: (B) MAP POSITION: D14S118 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:18: CAAACTCCTG AGGTCAAACA ATCC 2 4 (2) INFORMATION FOR SEQ ID NO:19: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 21 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($v\ i\ i\ i$) POSITION IN GENOME: (B) MAP POSITION: D14S562 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:19: CTTGGAGGGT GGGGTGGCTA A 2 1 (2) INFORMATION FOR SEQ ID NO:20: (i) SEOUENCE CHARACTERISTICS: (A) LENGTH: 24

47 48 -continued (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (v i i i) POSITION IN GENOME: (B) MAP POSITION: D14S562 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:20: CGAAATTTTG TTGCCTTGCT CTGG 2.4 (2) INFORMATION FOR SEQ ID NO:21: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: $21\,$ (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($\mathbf{v}\ \mathbf{i}\ \mathbf{i}\ \mathbf{i}$) POSITION IN GENOME: (B) MAP POSITION: D14S548 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:21: CCTGGGCAAC AGAGTGAGAC T 2 1 $(\ 2\)$ INFORMATION FOR SEQ ID NO:22: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (viii) POSITION IN GENOME: (B) MAP POSITION: D14S548 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:22: ACCCAGCTTT AACAGTTTGT GCTT 2 4 (2) INFORMATION FOR SEQ ID NO:23: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 23 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear $(\ v\ i\ i\ i\)$ Position in Genome: (B) MAP POSITION: D16S490 (\mathbf{x} i) SEQUENCE DESCRIPTION: SEQ ID NO:23: GGGCGGACAC AGAATGTAAA ATC 2 3 (2) INFORMATION FOR SEQ ID NO:24: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($\mathbf{v} \;\; \mathbf{i} \;\; \mathbf{i} \;\; \mathbf{i} \;\;)$ POSITION IN GENOME: (B) MAP POSITION: D16S490 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:24: AAACCCAAAT AGATGACAGG CACA 2 4 (2) INFORMATION FOR SEQ ID NO:25: (i) SEOUENCE CHARACTERISTICS: (A) LENGTH: 24

49 50 -continued (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (v i i i) POSITION IN GENOME: (B) MAP POSITION: D16S753 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:25: GCACTCCAGG CTGAATGACA GAAC 2.4 (2) INFORMATION FOR SEQ ID NO:26: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 $\,$ (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($\mathbf{v}\ \mathbf{i}\ \mathbf{i}\ \mathbf{i}$) POSITION IN GENOME: (B) MAP POSITION: D16S753 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:26: GCAGTGCCGC CTATTTTTGT GAAT $(\ 2\)$ INFORMATION FOR SEQ ID NO:27: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (viii) POSITION IN GENOME: (B) MAP POSITION: D17S1299 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:27: ACCCTGATGA GATAGCACTT GAGC 2 4 (2) INFORMATION FOR SEQ ID NO:28: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear $(\ v\ i\ i\ i\)$ Position in Genome: (B) MAP POSITION: D17S1299 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:28: CACTGTGTGG AGGTGTAGCA GAGA 2 4 (2) INFORMATION FOR SEQ ID NO:29: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($v\ i\ i\ i$) POSITION IN GENOME: (B) MAP POSITION: D16S539 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:29: GGGGGTCTAA GAGCTTGTAA AAAG 2 4 (2) INFORMATION FOR SEQ ID NO:30: (i) SEOUENCE CHARACTERISTICS: (A) LENGTH: 26

51 52 -continued (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (v i i i) POSITION IN GENOME: (B) MAP POSITION: D16S539 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:30: TGTGCATCTG TAAGCATGTA TCTATC 2 6 (2) INFORMATION FOR SEQ ID NO:31: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 23 $\,$ (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($\mathbf{v}\ \mathbf{i}\ \mathbf{i}\ \mathbf{i}$) POSITION IN GENOME: (B) MAP POSITION: D22S683 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:31: CGAAGGTTGC ATTGAGCCAA GAT $(\ 2\)$ INFORMATION FOR SEQ ID NO:32: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (viii) POSITION IN GENOME: (B) MAP POSITION: D22S683 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:32: TGGTGGAAAT GCCTCATGTA GAAA 2 4 (2) INFORMATION FOR SEQ ID NO:33: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear $(\ v\ i\ i\ i\)$ Position in Genome: (B) MAP POSITION: HUMCSF1PO (x i) SEQUENCE DESCRIPTION: SEQ ID NO:33: AACCTGAGTC TGCCAAGGAC TAGC 2 4 (2) INFORMATION FOR SEQ ID NO:34: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($v\ i\ i\ i$) POSITION IN GENOME: ($\, B \,$) MAP POSITION: HUMCSF1PO (x i) SEQUENCE DESCRIPTION: SEQ ID NO:34: TTCCACACAC CACTGGCCAT CTTC 2 4 (2) INFORMATION FOR SEQ ID NO:35: (i) SEOUENCE CHARACTERISTICS: (A) LENGTH: 24

53 54 -continued (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (v i i i) POSITION IN GENOME: (B) MAP POSITION: HUMTPOX (x i) SEQUENCE DESCRIPTION: SEQ ID NO:35: ACTGGCACAG AACAGGCACT TAGG 2.4 (2) INFORMATION FOR SEQ ID NO:36: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 $\,$ (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($\mathbf{v}\ \mathbf{i}\ \mathbf{i}\ \mathbf{i}$) POSITION IN GENOME: ($\, B \,$) MAP POSITION: HUMTPOX (x i) SEQUENCE DESCRIPTION: SEQ ID NO:36: GGAGGAACTG GGAACCACAC AGGT $(\ 2\)$ INFORMATION FOR SEQ ID NO:37: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (viii) POSITION IN GENOME: (B) MAP POSITION: HUMTHO1 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:37: ATTCAAAGGG TATCTGGGCT CTGG 2 4 (2) INFORMATION FOR SEQ ID NO:38: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear $(\ v\ i\ i\ i\)$ Position in Genome: (B) MAP POSITION: HUMTHO1 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:38: GTGGGCTGAA AAGCTCCCGA TTAT 2 4 (2) INFORMATION FOR SEQ ID NO:39: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 29 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($v\ i\ i\ i$) POSITION IN GENOME: (B) MAP POSITION: $\mbox{HUMvWFA31}$ (x i) SEQUENCE DESCRIPTION: SEQ ID NO:39: GAAAGCCCTA GTGGATGATA AGAATAATC 29 (2) INFORMATION FOR SEQ ID NO:40: (i) SEOUENCE CHARACTERISTICS: (A) LENGTH: 30

55 56 -continued (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (v i i i) POSITION IN GENOME: (B) MAP POSITION: HUMvWFA31 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:40: GGACAGATGA TAAATACATA GGATGGATGG 3 0 (2) INFORMATION FOR SEQ ID NO:41: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 $\,$ (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($\mathbf{v}\ \mathbf{i}\ \mathbf{i}\ \mathbf{i}$) POSITION IN GENOME: (B) MAP POSITION: HUMF13A01 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:41: GAGGTTGCAC TCCAGCCTTT GCAA $(\ 2\)$ INFORMATION FOR SEQ ID NO:42: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (viii) POSITION IN GENOME: (B) MAP POSITION: HUMF13A01 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:42: TTCCTGAATC ATCCCAGAGC CACA 2 4 (2) INFORMATION FOR SEQ ID NO:43: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 23 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear $(\ v\ i\ i\ i\)$ Position in Genome: (B) MAP POSITION: HUMFESFPS (x i) SEQUENCE DESCRIPTION: SEQ ID NO:43: GCTGTTAATT CATGTAGGGA AGG 2 3 (2) INFORMATION FOR SEQ ID NO:44: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($v\ i\ i\ i$) POSITION IN GENOME: $(\ B\)$ MAP POSITION: HUMFESFPS (x i) SEQUENCE DESCRIPTION: SEQ ID NO:44: GTAGTCCCAG CTACTTGGCT ACTC 2 4 (2) INFORMATION FOR SEQ ID NO:45: (i) SEOUENCE CHARACTERISTICS: (A) LENGTH: 20

57 58 -continued (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (v i i i) POSITION IN GENOME: (B) MAP POSITION: HUMBFXIII (x i) SEQUENCE DESCRIPTION: SEQ ID NO:45: TGAGGTGGTG TACTACCATA 2 0 (2) INFORMATION FOR SEQ ID NO:46: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: $20\,$ (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($\mathbf{v}\ \mathbf{i}\ \mathbf{i}\ \mathbf{i}$) POSITION IN GENOME: (B) MAP POSITION: HUMBFXIII (x i) SEQUENCE DESCRIPTION: SEQ ID NO:46: GATCATGCCA TTGCACTCTA 2 0 $(\ 2\)$ INFORMATION FOR SEQ ID NO:47: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (viii) POSITION IN GENOME: (B) MAP POSITION: HUMLIPOL $(\ x\ i\)$ SEQUENCE DESCRIPTION: SEQ ID NO:47: CTGACCAAGG ATAGTGGGAT ATAG 2 4 (2) INFORMATION FOR SEQ ID NO:48: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 23 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($\,{\bf v}\,\,\,{\bf i}\,\,\,{\bf i}\,\,\,{\bf i}\,\,\,)$ POSITION IN GENOME: (B) MAP POSITION: HUMLIPOL (x i) SEQUENCE DESCRIPTION: SEQ ID NO:48: GGTAACTGAG CGAGACTGTG TCT 2 3 (2) INFORMATION FOR SEQ ID NO:49: $(\ \ i\)$ SEQUENCE CHARACTERISTICS: (A) LENGTH: 18 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (viii) POSITION IN GENOME: (B) MAP POSITION: D3S1539 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:49: CCACCCTTTC AGCACCAG (2) INFORMATION FOR SEQ ID NO:50: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 20 (B) TYPE: Nucleic Acid

59 60 -continued (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear $(\ v\ i\ i\ i\)$ POSITION IN GENOME: (B) MAP POSITION: D19S253 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:50: ATAGACAGAC AGACGGACTG 2 0 (2) INFORMATION FOR SEQ ID NO:51: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 19 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (viii) POSITION IN GENOME: (B) MAP POSITION: D19S253 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:51: GGGAGTGGAG ATTACCCCT 19 (2) INFORMATION FOR SEQ ID NO:52: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 21 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (v i i i) POSITION IN GENOME: (B) MAP POSITION: D20S481 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:52: AAAGCTCTCT GAAGCAGGTG T 2 1 (2) INFORMATION FOR SEQ ID NO:53: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($\,{\bf v}\,\,\,{\bf i}\,\,\,{\bf i}\,\,\,{\bf i}\,\,\,)$ POSITION IN GENOME: (B) MAP POSITION: D20S481 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:53: CAGATTGCAC TAGAAAGAGA GGAA 2 4 (2) INFORMATION FOR SEQ ID NO:54: $(\ \ i\)$ SEQUENCE CHARACTERISTICS: (A) LENGTH: 23 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (viii) POSITION IN GENOME: (B) MAP POSITION: D10S1239 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:54: CACCCTGTCC CCAGCTATCT GGA 2 3 (2) INFORMATION FOR SEQ ID NO:55: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 27 (B) TYPE: Nucleic Acid

61 62 -continued (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear $(\ v\ i\ i\ i\)$ POSITION IN GENOME: (B) MAP POSITION: D9S930 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:55: AGTTGAATCT TGAGTCTCTC AGAGTCA 2 7 (2) INFORMATION FOR SEQ ID NO:56: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (viii) POSITION IN GENOME: ($\,B\,$) MAP POSITION: D4S2368 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:56: TGTACTCATT TTCCCGCAAT GATG 2 4 $(\ 2\)$ INFORMATION FOR SEQ ID NO:57: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (v i i i) POSITION IN GENOME: (B) MAP POSITION: D4S2368 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:57: TCAGAAAGTA GGGTCTGGGC TCTT 2 4 (2) INFORMATION FOR SEQ ID NO:58: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 28 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear ($\,{\bf v}\,\,\,{\bf i}\,\,\,{\bf i}\,\,\,{\bf i}\,\,\,)$ POSITION IN GENOME: (B) MAP POSITION: D16S539 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:58: TGTGCATCTG TAAGCATGTA TCTATCAT 2 8 (2) INFORMATION FOR SEQ ID NO:59: $(\ \ i\)$ SEQUENCE CHARACTERISTICS: (A) LENGTH: 32 (B) TYPE: Nucleic Acid (C) STRANDEDNESS: Single (D) TOPOLOGY: Linear (viii) POSITION IN GENOME: (B) MAP POSITION: HUMvWFA31 (x i) SEQUENCE DESCRIPTION: SEQ ID NO:59: GAAAGCCCTA GTGGATGATA AGAATAATCA GT 3 2 (2) INFORMATION FOR SEQ ID NO:60: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 33 (B) TYPE: Nucleic Acid

-continued 64

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(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(viii) POSITION IN GENOME:

(B) MAP POSITION: HUMvWFA31

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GGACAGATGA TAAATACATA GGATGGATGG ATA

3 3

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(viii) POSITION IN GENOME:

(B) MAP POSITION: D9S930

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:61:

GCTATGGGAA TTACAAGCAG GAAAC

2 5

What is claimed is:

- 1. A method of simultaneously determining the alleles present in at least four short tandem repeat loci from one or more DNA samples, comprising:
 - (a) obtaining at least one DNA sample to be analyzed,
 - (b) selecting a set of at least four short tandem repeat loci of the DNA sample to be analyzed which can be amplified together, wherein the at least four loci in the set are selected from the group of loci consisting of: D3S1539, D4S2368, D5S818, D7S820, D9S930, 35

D10S1239, D13S317, D14S118, D14S548, D14S562, D16S490, D16S539, D16S753, D17S1298, D17S1299, D19S253, D2OS481, D22S683, HUMCSF1PO, HUMTPOX, HUMTH01, HUMF13A01, HUMBFXIII, HUMLIPOL, HUM-vWFA31;

- (c) co-amplifying the loci in the set in a multiplex amplification reaction, wherein the product of the reaction is a mixture of amplified alleles from each of the co-amplified loci in the set; and
- (d) evaluating the amplified alleles in the mixture to determine the alleles present at each of the loci analyzed in the set within the DNA sample.
- 2. The method of claim 1, wherein the set of at least four loci co-amplified therein is a set of four loci, wherein the set 50 of four loci is selected from the group of sets of loci consisting of:

D3S1539, D7S820, D13S317, D5S818; D17S1298, D7S820, D13S317, D5S818; D2OS481, D7S820, D13S317, D5S818; D9S930, D7S820, D13S317, D5S818; D10S1239, D7S820, D13S317, D5S818; D14S118, D7S820, D13S317, D5S818; D14S562, D7S820, D13S317, D5S818; D14S548, D7S820, D13S317, D5S818; D14S549, D7S820, D13S317, D5S818; D16S490, D7S820, D13S317, D5S818; D17S1299, D7S820, D13S317, D5S818; D16S539, D7S820, D13S317, D5S818; D2S683, D7S820, D13S317, D5S818; D2S683, D7S820, D13S317, D5S818; D16S753, D7S820, D13S317, D5S818;

- D3S1539, D19S253, D13S317, D20S481; D3S1539, D19S253, D4S2368, D20S481; D10S1239, D9S930, D4S2368, D20S481; and D16S539, D7S820, D13S317, HUMvWFA31.
- 3. The method of claim 1, wherein the set of at least four loci co-amplified therein is a set of six loci, wherein the set of six loci is selected from the group of sets of loci consisting of:
 - D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, HUMTPOX; and
 - D16S539, D7S820, D13S317, D5S818, HUMF13A01, HUMFESFPS.
- 4. The method of claim 1, wherein the set of at least four loci co-amplified therein is a set of seven loci, wherein the set is selected from the group of sets of loci consisting of:
 - D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, HUMTPOX, HUMTH01; and
 - D16S539, D7S820, D13S317, D5S818, HUMF13A01, HUMFESFPS, HUMBFXIII.
- 5. The method of claim 1, wherein the set of at least four loci co-amplified therein is a set of at least eight loci, and wherein the set is selected from the group of sets of loci consisting of:
 - D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, HUMTPOX, HUMTH01, HUMvWFA31; and
 - D16S539, D7S820, D13S317, D5S818, HUMF13A01, HUMFESFPS, HUMBFXIII, HUMLIPOL.
- 6. The method of claim 1, wherein the multiplex amplification reaction is done using at least four pair of primers flanking the at least four loci analyzed.
- 7. The method of claim 6, additionally comprising the step of selecting pairs of primers for the multiplex amplification reaction which produce alleles from each locus that do not overlap the alleles of the other loci in the set co-amplified therein, when the alleles are separated by gel electrophoresis.
- 8. The method of claim 6, wherein at least one of each of the pairs of primers used in the multiplex amplification reaction has a sequence selected from one of the groups of sequences consisting of:

SEQ ID NO:1 and SEQ ID NO:2, when one of the loci in the set is D7S820;

- SEQ ID NO:3 and SEQ ID NO:4, when one of the loci in the set is D13S317;
- SEQ ID NO:5 and SEQ ID NO:6, when one of the loci in the set is D5S818:
- SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:49, when one of the loci in the set is D3S1539;
- SEQ ID NO:9, SEQ ID NO:10, when one of the loci in the set is D17S1298;
- SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:52, SEQ ID NO:53, when one of the loci in the set is D20S481;
- SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:55, SEQ ID NO:61, when one of the loci in the set is D9S930;
- SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:54, when one of the loci in the set is D10S1239;
- SEQ ID NO:17, SEQ ID NO:18, when one of the loci in the set is D14S118;
- SEQ ID NO:19, SEQ ID NO:20, when one of the loci in the set is D14S562;
- SEQ ID NO:21, SEQ ID NO:22, when one of the loci in the set is D14S548;
- SEQ ID NO:23, SEQ ID NO:24, when one of the loci in the set is D16S490;
- SEQ ID NO:25, SEQ ID NO:26, when one of the loci in $_{\ \, 25}$ the set is D16S753;
- SEQ ID NO:27, SEQ ID NO:28, when one of the loci in the set is D17S1299;
- SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:58, when one of the loci in the set is D16S539;
- SEQ ID NO:31, SEQ ID NO:32, when one of the loci in the set is D22S683;
- SEQ ID NO:33, SEQ ID NO:34, when one of the loci in the set is HUMCSF1PO;
- SEQ ID NO:35, SEQ ID NO:36, when one of the loci in 35 the set is HUMTPOX;
- SEQ ID NO:37, SEQ ID NO:38, when one of the loci in the set is HUMTH01;
- SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:59, SEQ ID NO:60 when one of the loci in the set is HUMvWFA31;
- SEQ ID NO:41, SEQ ID NO:42, when one of the loci in the set is HUMF13A01;
- SEQ ID NO:43, SEQ ID NO:44, when one of the loci in the set is HUMFESFPS;
- SEQ ID NO:45, SEQ ID NO:46, when one of the loci in the set is HUMBFXIII;
- SEQ ID NO:47, SEQ ID NO:48, when one of the loci in the set is HUMLIPOL;
- SEQ ID NO:50, SEQ ID NO:51, when one of the loci in $_{50}$ the set is D19S253; and
- SEQ ID NO:56, SEQ ID NO:57, when one of the loci in the set is D4S2368.
- **9**. The method of claim **6**, wherein the multiplex amplification reaction is a polymerase chain reaction.
- 10. The method of claim 1, wherein the amplified alleles are evaluated by comparing the amplified alleles to a size standard, wherein the size standard is selected from the group of size standards consisting of a DNA marker and a locus-specific allelic ladder.
- 11. The method of claim 1, wherein the amplified alleles are evaluated using polyacrylamide gel electrophoresis to separate the alleles, forming a polyacrylamide gel of separated alleles.
- 12. The method of claim 11, wherein the separated alleles 65 in the polyacrylamide gel are determined by visualizing the alleles with silver stain analysis.

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- 13. The method of claim 11, wherein primers capable of binding to a region flanking each of the loci in the set are used in co-amplifying the loci, wherein at least one of the primers used in co-amplifying each locus has a fluorescent label covalently attached thereto such that the amplified alleles produced therefrom are fluorescently labeled, and wherein the separated alleles in the polyacrylamide gel are determined by visualizing the alleles with fluorescent analysis
- 14. The method of claim 13, wherein the fluorescent label is selected from the group of labels consisting of fluorescein and tetramethyl rhodamine.
- 15. The method of claim 1 wherein the at least one DNA sample to be analyzed is isolated from human tissue, wherein the human tissue is selected from the group of human tissue consisting of blood, semen, vaginal cells, hair, saliva, urine, amniotic fluid containing placental cells or fetal cells, and mixtures of any of the tissues listed above.
 - 16. A method of simultaneously determining the alleles present in three short tandem repeat loci from one or more DNA samples, comprising:
 - (a) obtaining at least one DNA sample to be analyzed,
 - (b) selecting a set of three short tandem repeat loci of the DNA sample to be analyzed which can be amplified together, wherein the set of three loci is selected from the group of sets of loci consisting of:

D3S1539, D19S253, D13S317;

D10S1239, D9S930, D20S481;

D10S1239, D4S2368, D20S481;

D10S1239, D9S930, D4S2368;

D16S539, D7S820, D13S317; and

D10S1239, D9S930, D13S317.

- (c) co-amplifying the three loci in the set in a multiplex amplification reaction, wherein the product of the reaction is a mixture of amplified alleles from each of the co-amplified loci in the set; and
- (d) evaluating the amplified alleles in the mixture to determine the alleles present at each of the loci analyzed in the set within the DNA sample.
- 17. The method of claim 16, wherein the multiplex amplification reaction is done using three pair of primers, wherein each pair of primers flanks one of the three short tandem repeat loci in the set of loci co-amplified in the reaction.
- 18. The method of claim 17, wherein each of the three pair of primers used in the multiplex amplification reaction is designed to hybridize with an allele of a locus in the set of loci co-amplified in the reaction, wherein:
 - when D7S820 is one of the loci in the set of loci co-amplified, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2;
 - when D13S317 is one of the loci in the set of loci co-amplified, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:3 and SEQ ID NO:4;
 - when D2OS481 is one of the loci in the set of loci co-amplified, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:52, SEQ ID NO:53;
 - when D9S930 is one of the loci in the set of loci co-amplified, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:55 and SEQ ID NO:61;
 - when D10S1239 is one of the loci in the set of loci co-amplified, at least one of the primers has a sequence

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- selected from the group consisting of SEQ ID NO:15, SEQ ID NO:16 and SEQ ID NO:44;
- when D16S539 is one of the loci in the set of loci co-amplified, at least one of the primers has a sequence selected from the group consisting of SEQ ID $\hat{N}O:29^{-5}$ and SEQ ID NO:30; and
- when D4S2368 is one of the loci in the set of loci co-amplified, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:56 and SEQ ID NO:57.
- 19. The method of claim 16, wherein the multiplex amplification reaction is a polymerase chain reaction.
- 20. The method of claim 16, wherein the amplified alleles are evaluated by comparing the amplified alleles to a size standard, wherein the size standard is selected from the group of size standards consisting of a DNA marker and a locus-specific allelic ladder.
- 21. The method of claim 16, wherein the amplified alleles are evaluated using polyacrylamide gel electrophoresis to separate the alleles, forming a polyacrylamide gel of sepa- 20 rated alleles.
- 22. The method of claim 21, wherein the separated alleles in the polyacrylamide gel are determined by visualizing the alleles with silver stain analysis.
- 23. The method of claim 21, wherein the separated alleles ²⁵ in the polyacrylamide gel are determined by visualizing the alleles with fluorescence analysis.
- 24. The method of claim 16 wherein the at least one DNA sample to be analyzed is isolated from human tissue, wherein the human tissue is selected from the group of 30 human tissue consisting of blood, semen, vaginal cells, hair, saliva, urine, bone, buccal sample, amniotic fluid containing placental cells or fetal cells, and mixtures of any of the tissues listed above.
- 25. A kit for simultaneously analyzing short tandem 35 repeat sequences in at least three loci, comprising a container which has oligonucleotide primers for co-amplifying a set of at least three short tandem repeat loci, wherein the set of loci are selected from the sets of loci consisting of:

D3S1539, D19S253, D13S317;

D10S1239, D9S930, D20S481;

D10S1239, D4S2368, D20S481;

D10S1239, D9S930, D4S2368;

D16S539, D7S820, D13S317;

D10S1239, D9S930, D13S317;

D3S1539, D7S820, D13S317, D5S818;

D17S1298, D7S820, D13S317, D5S818;

D20S481, D7S820, D13S317, D5S818;

D9S930, D7S820, D13S317, D5S818;

D10S1239, D7S820, D13S317, D5S818;

D14S118, D7S820, D13S317, D5S818;

D14S562, D7S820, D13S317, D5S818;

D14S548, D7S820, D13S317, D5S818;

D16S490, D7S820, D13S317, D5S818;

D17S1299, D7S820, D13S317, D5S818;

D16S539, D7S820, D13S317, D5S818;

D22S683, D7S820, D13S317, D5S818;

D16S753, D7S820, D13S317, D5S818;

D3S1539, D19S253, D13S317, D20S481;

D3S1539, D19S253, D4S2368, D20S481;

D10S1239, D9S930, D4S2368, D20S481;

D16S539, D7S820, D13S317, HUMvWFA31;

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- D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, HUMTPOX;
- D16S539, D7S820, D13S317, D5S818, HUMF13A01, HUMFESFPS;
- D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, HUMTPOX, HUMTH01;
- D16S539, D7S820, D13S317, D5S818, HUMF13A01, HUMFESFPS, HUMBFXIII;
- D16S539, D7S820, D13S317, D5S818, HUMCSF1PO, HUMTPOX, HUMTH01, HUMvWFA31; and
- D16S539, D7S820, D13S317, D5S818, HUMF13A01, HUMFESFPS, HUMBFXIII, HUMLIPOL.
- 26. The kit of claim 25, wherein each of the oligonucle-15 otide primers is designed to hybridize with an allele of one of the three loci in the set of loci selected, wherein:
 - when D7S820 is one of the loci in the set, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2;
 - when D13S317 is one of the loci in the set, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:3 and SEQ ID NO:4;
 - when D5S818, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:5 and SEQ ID NO:6;
 - when D3S153, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:7, SEQ ID NO:8 and SEQ ID NO:49;
 - when D17S1298, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:9 and SEQ ID NO:10;
 - when D20S481, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:52, SEQ ID NO:53;
 - when D9S930, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:55, SEQ ID NO:61;
 - when D10S1239, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:54;
 - when D14S118, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:17, SEQ ID NO:18;
 - when D14S562, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:19, SEO ID NO:20;
 - when D14S548, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:21, SEQ ID NO:22;
 - when D16S490, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24;
 - when D16S753, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:25, SEQ ID NO:26;
 - when D17S1299, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:27, SEQ ID NO:28;
 - when D16S539, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:58;
 - when D22S683, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:31, SEQ ID NO:32;

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- when HUMCSF1PO, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:33, SEQ ID NO:34;
- when HUMTPOX, at least one of the primers has a sequence selected from the group consisting of SEQ ID 5 NO:35; SEQ ID NO:36;
- when HUMTH01, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:37, SEQ ID NO:38;
- when HUMvWFA31, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:60;
- when HUMF13A01, at least one of the primers has a NO:41, SEQ ID NO:42;
- when HUMFESFPS, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:43, SEQ ID NO:44;
- when HUMBFXIII, at least one of the primers has a 20 sequence selected from the group consisting of SEQ ID NO:45, SEQ ID NO:46;
- when HUMLIPOL, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:47, SEQ ID NO:48;
- when D19S253, at least one of the primers has a sequence selected from the group consisting of SEQ ID NO:50, SEQ ID NO:51; and
- when D4S2368, at least one of the primers has a sequence 30 selected from the group consisting of SEQ ID NO:56, SEQ ID NO:57.
- 27. The kit of claim 25, further comprising a container having reagents for at least one multiplex amplification
- 28. The kit of claim 25, further comprising a container having an allelic ladder.
- 29. The kit of claim 28, wherein each rung of the allelic ladder and at least one oligonucleotide primer for each of the loci in the set each have a label covalently attached thereto. 40
- 30. The kit of claim 29, wherein the label is a fluorescent label.
- 31. The kit of claim 30, wherein at least one of the oligonucleotide primers has a different fluorescent label covalently attached thereto than some of the other primer 45 pairs in the container.
- 32. A method of simultaneously determining the alleles present in at least four short tandem repeat loci from one or more DNA samples, comprising:
 - (a) obtaining at least one DNA sample to be analyzed,
 - (b) selecting a set of at least four short tandem repeat loci of the DNA sample to be analyzed which can be amplified together, wherein three of the loci in the set are D7S820, D13S317, and D5S818;

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- (c) co-amplifying the loci in the set in a multiplex amplification reaction, wherein the product of the reaction is a mixture of amplified alleles from each of the co-amplified loci in the set; and
- (d) evaluating the amplified alleles in the mixture to determine the alleles present at each of the loci analyzed in the set within the DNA sample.
- 33. The method of claim 32, wherein the multiplex amplification reaction is done using at least four pair of primers flanking the at least four loci analyzed.
- 34. The method of claim 33, additionally comprising the step of selecting pairs of primers for the multiplex amplification reaction which produce alleles from each locus that sequence selected from the group consisting of SEQ ID 15 do not overlap the alleles of the other loci in the set co-amplified therein, when the alleles are separated by gel electrophoresis.
 - 35. The method of claim 32, wherein the multiplex amplification reaction is a polymerase chain reaction.
 - 36. The method of claim 32, wherein the amplified alleles are evaluated by comparing the amplified alleles to a size standard, wherein the size standard is selected from the group of size standards consisting of a DNA marker and a locus-specific allelic ladder.
 - 37. The method of claim 32, wherein the amplified alleles are evaluated using polyacrylamide gel electrophoresis to separate the alleles, forming a polyacrylamide gel of separated alleles.
 - 38. The method of claim 37, wherein the separated alleles in the polyacrylamide gel are determined by visualizing the alleles with silver stain analysis.
 - 39. The method of claim 37, wherein primers capable of binding to a region flanking each of the loci in the set are used in co-amplifying the loci, wherein at least one of the primers used in co-amplifying each locus has a fluorescent label covalently attached thereto such that the amplified alleles produced therefrom are fluorescently labeled, and wherein the separated alleles in the polyacrylamide gel are determined by visualizing the alleles with fluorescence analysis.
 - 40. The method of claim 39, wherein the fluorescent label is selected from the group of labels consisting of fluorescein and tetramethyl rhodamine.
 - 41. The method of claim 6, wherein one of each of at the least four pair of primers used in the multiplex amplification reaction has a fluorescent label covalently attached thereto.
 - 42. The method of claim 41, wherein at least four of the primers used in the multiplex amplification reaction have the same fluorescent label covalently attached thereto.
 - 43. The kit of claim 30, wherein at least four of the oligonucleotide primers have the same fluorescent label covalently attached thereto.

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TAB 7

US006221598B1

(12) United States Patent

Schumm et al.

(10) Patent No.: US 6,221,598 B1

(45) **Date of Patent:** *Apr. 24, 2001

(54) MULTIPLEX AMPLIFICATION OF SHORT TANDEM REPEAT LOCI

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0.5.c. 15 1(b) by 6 days.

This patent is subject to a terminal dis-

claimer.

(21) Appl. No.: 09/327,229

(22) Filed: Jun. 7, 1999

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(63) Continuation of application No. 08/316,544, filed on Sep. 30, 1994, now abandoned.

(51) **Int. Cl.**⁷ **C12Q 1/70**; C07H 21/04

(52) **U.S. Cl.** **435/6**; 435/91.1; 435/91.2; 536/23.1; 536/24.31; 536/24.33

330/23.1; 330/24.31; 330/24.33

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7, 8, 11, 15, 16, 19, 20, 27, and 28.*

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(57) ABSTRACT

The present invention is directed to the simultaneous amplification of multiple distinct genetic loci using PCR or other amplification systems to determine in one reaction the alleles of each locus contained within the multiplex.

43 Claims, 7 Drawing Sheets

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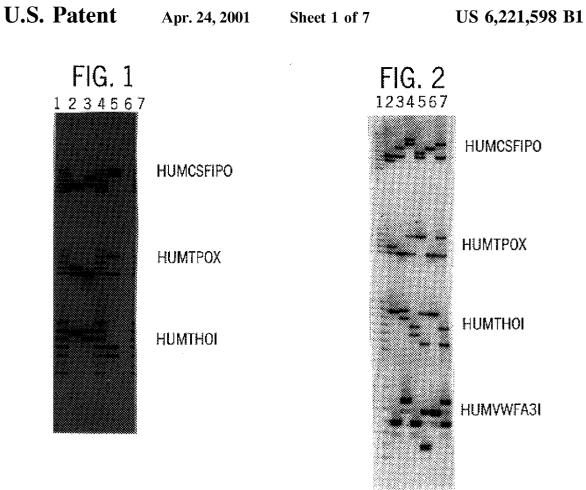
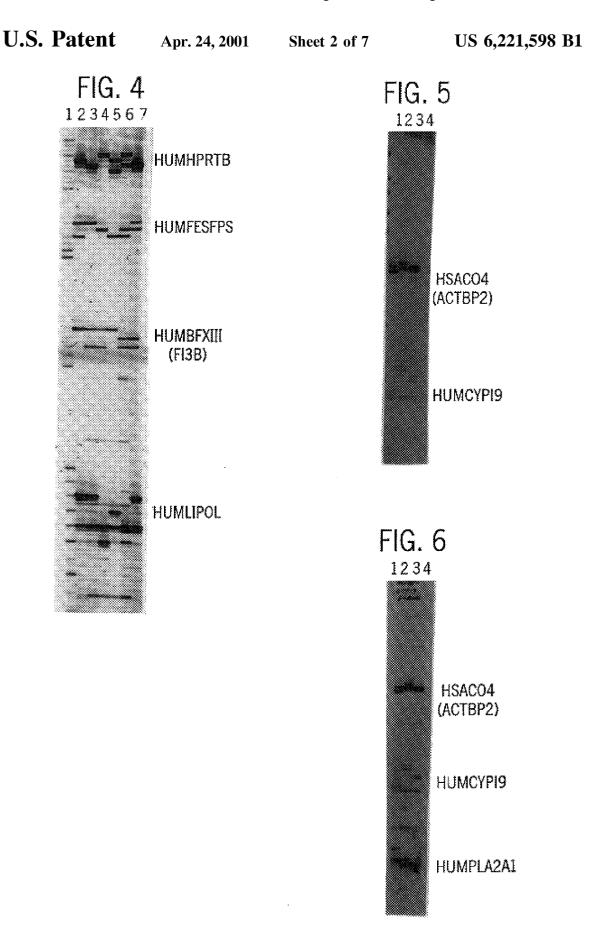


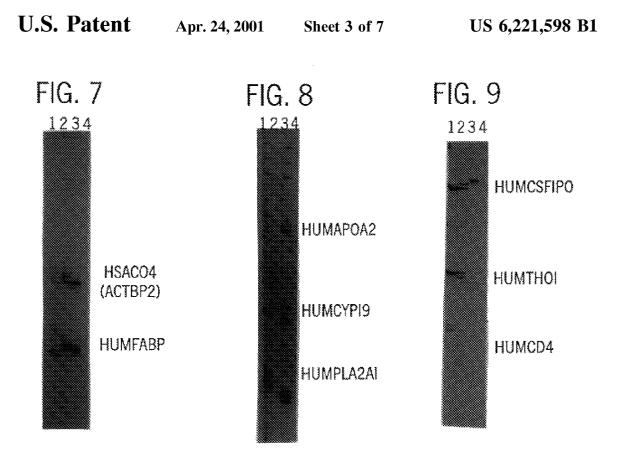
FIG. 3
1234567

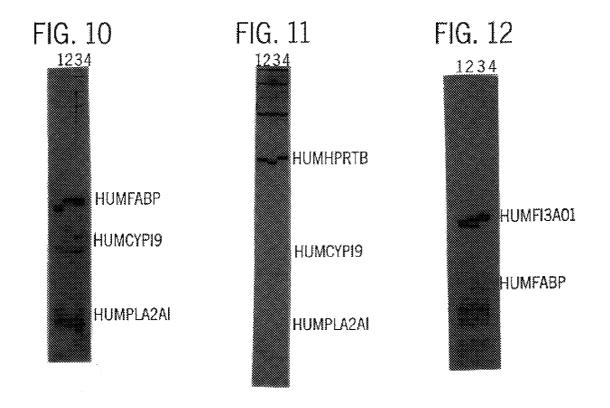
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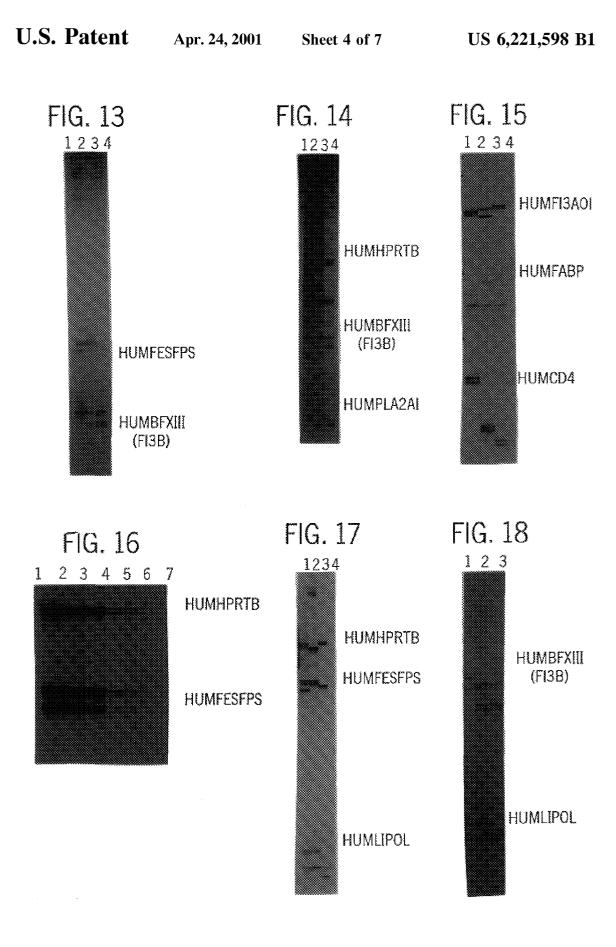
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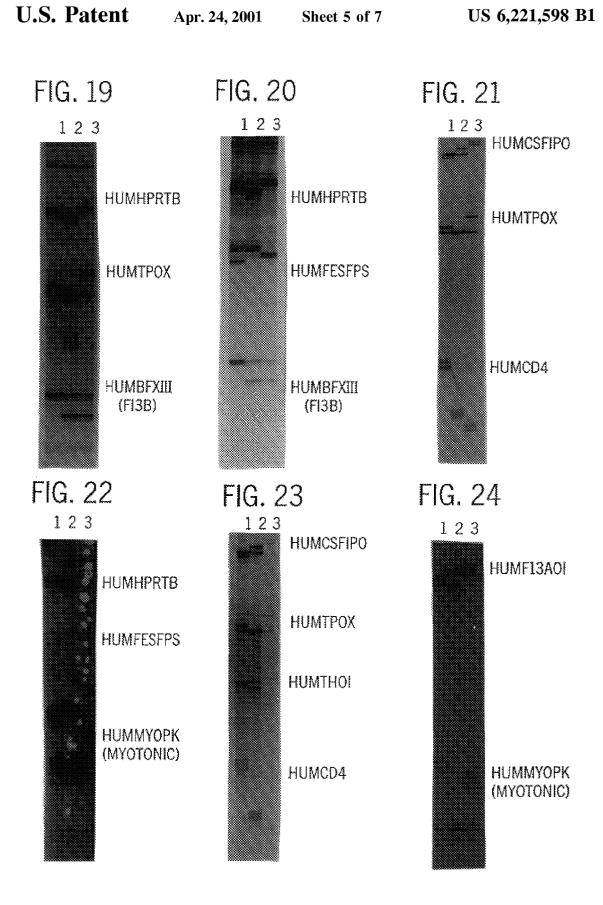
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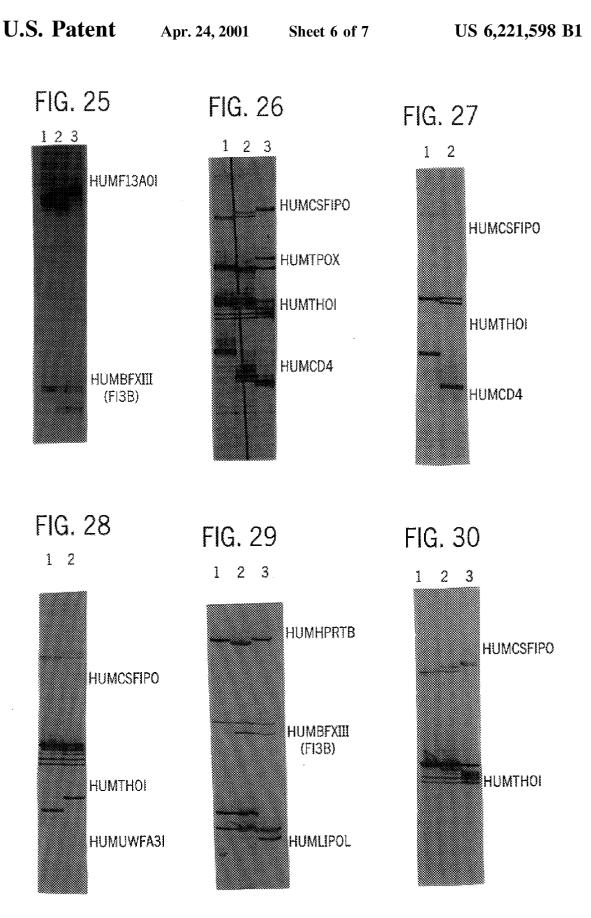












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FIG. 31

2 3 1

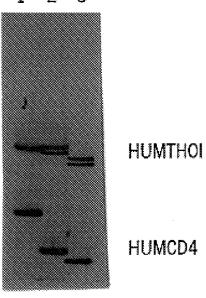
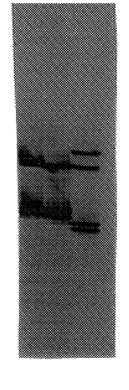


FIG. 32

1 2 3



HUMTPOX

HUMTHOL

1

MULTIPLEX AMPLIFICATION OF SHORT TANDEM REPEAT LOCI

CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation of U.S. patent application Ser. No. 08/316,544, filed Sep. 30, 1994, now abandoned.

FIELD OF THE INVENTION

The present invention is generally directed to the detection of genetic markers in a genomic system. The present invention is more specifically directed to the simultaneous amplification of multiple distinct polymorphic genetic loci using the polymerase chain reaction or other amplification systems to determine in one reaction the alleles of each locus contained within the multiplex system.

CITED REFERENCES

A full bibliographic citation of the references cited in this application can be found in the section preceding the claims.

DESCRIPTION OF THE PRIOR ART

In recent years, the discovery and development of polymorphic short tandem repeats (STRs) as genetic markers has stimulated progress in the development of linkage maps, the identification and characterization of diseased genes, and the simplification and precision of DNA typing.

Many loci, at least in the human genome, contain a polymorphic STR region. STR loci consist of short, repetitive sequence elements of 3 to 7 base pairs in length. It is estimated that there are 2,000,000 expected trimeric and tetrameric STRs present as frequently as once every 15 kilobases (kb) in the human genome (Edwards et al. 1991; Beckmann and Weber 1992). Nearly half of the STR loci studied by Edwards et al. (1991) are polymorphic, which provides a rich source of genetic markers. Variation in the number of repeat units at a particular locus is responsible for the observed polymorphism reminiscent of VNTR loci (Nakamura et al. 1987) and minisatellite loci (Jeffreys et al. 1985), which contain longer repeat units, and microsatellite or dinucleotide repeat loci (Litt and Luty 1989, Tautz 1989, Weber and May 1989, Beckmann and Weber 1992).

Polymorphic STR loci are extremely useful markers for human identification, paternity testing and genetic mapping. STR loci may be amplified via the polymerase chain reaction (PCR) by employing specific primer sequences identified in the regions flanking the tandem repeat.

Alleles of these loci are differentiated by the number of copies of the repeat sequence contained within the amplified region and are distinguished from one another following electrophoretic separation by any suitable detection method including radioactivity, fluorescence, silver stain, and color. 55

To minimize labor, materials and analysis time, it is desirable to analyze multiple loci and/or more samples simultaneously. One approach for reaching this goal involves amplification of multiple loci simultaneously in a single reaction. Such "multiplex" amplifications have been described extensively in the literature. Multiplex amplification sets have been extensively developed for analysis of genes related to human genetic diseases such as Duchenne Muscular Dystrophy (Chamberlain et al. 1988, Chamberlain et al. 1989, Beggs et al. 1990, Clemens et al. 1991, Schwartz et al. 1992, Covone et al. 1992), Lesch-Nyhan Syndrome (Gibbs et al. 1990), Cystic Fibrosis (Estivill et al. 1991,

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Fortina et al. 1992, Ferrie et al. 1992, Morral and Estivill, 1992), and Retinoblasma (Lohmann et al. 1992). Multiplex amplification of polymorphic microsatellite markers (Clemens et al. 1991, Schwartz et al. 1992, Huang et al. 1992) and even STR markers (Edwards et al. 1992, Kimpton et al. 1993, Hammond et al. 1994) have been described.

These amplified products are generally separated by one of several methods of electrophoresis known to those skilled in the art. Several well-known methods of detection of the 10 amplified products have also been described. While ethidium bromide staining of amplified fragments is employed in some cases, in others it is preferred to use methods which label only one of the two strands of the amplified material. Examples of this include radioactive or 15 fluorescent labeling of one of the two primers prior to the amplification of a locus. One of the more sophisticated approaches to detection is the use of different fluorescent labels to allow detection of amplified materials representing different loci, but existing in the same space following electrophoresis. The products of the different loci are differentiated with the use of filters, which allow visualization of one fluorescent label at a time.

Reference is made to International Publications WO 93/18177 and WO 93/18178 to Fortina et al., which are directed to methods and kits for diagnosing diseases such as Cystic Fibrosis and β -thalassemia, respectively, using an allele-specific multiplex polymerase chain reaction system. According to Fortina et al., multiplex PCR has also been used for simultaneous amplification of multiple target sequences, permitting mutant allele scanning using two lanes of an agarose gel.

Ballabio et al. (1991), disclose a single-tube, multiplex allele specific PCR test using two different dye-tagged fluorescent primers for detection of the ▲F508 cystic fibrosis mutation.

While there are multiplex amplification procedures for specific loci, the use of multiplex amplification procedures is greatly desired for the detection of alleles in other types of loci such as specific STR loci.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a method for the simultaneous amplification of multiple distinct polymorphic STR loci using PCR or other amplification systems to determine, in one reaction, the alleles of each locus contained within the multiplex. These combinations of specific loci into multiplexes have not been heretofore shown.

It is also an object of the present invention to provide a method and a kit specific for multiplex amplifications comprising specified loci.

These and other objects are addressed by the present invention which is directed to a method of simultaneously analyzing or determining the alleles present at each individual locus of each multiplex. This method comprises the steps of (1) obtaining at least one DNA sample to be analyzed, wherein the DNA sample has at least two loci which can be amplified together; (2) amplifying the STR sequences in the DNA sample; and (3) detecting the amplified materials in a fashion which reveals the polymorphic nature of the systems employed.

genes related to human genetic diseases such as Duchenne Muscular Dystrophy (Chamberlain et al. 1988, Chamberlain et al. 1989, Beggs et al. 1990, Clemens et al. 1991, Schwartz 65 at least one of the loci is selected from the group consisting et al. 1992, Covone et al. 1992), Lesch-Nyhan Syndrome (Gibbs et al. 1990), Cystic Fibrosis (Estivill et al. 1991, HUMCSF1PO, HUMTPOX, HUMVWFA31, HUMFESFPS, HUMBFXIII (F13B), HUMLIPOL,

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HSAC04 (ACTBP2), HUMCYP19, HUMPLA2A1, HUMAPOA2, HUMCD4, HUMF13A01 and HUM-MYOPK (Myotonic).

Specifically, the present invention is directed to a method of simultaneously analyzing multiple STR sequences in the 5 following groups of loci: HUMTH01 and HUMCSF1PO; HUMTH01 and HUMCD4; HUMTH01 and HUMTPOX; HUMF13A01 and HUMFABP; HUMF13A01 and HUM-MYOPK (Myotonic); HUMF13A01 and HUMBFXIII (F13B); HUMBFXIII (F13B) and HUMFESFPS; 10 HUMBFXIII (F13B) and HUMLIPOL; HUMHPRTB and HUMFESFPS; HSAC04 (ACTBP2) and HUMCYP19; HUMCSF1PO, HUMTPOX and HUMTH01; HUMHPRTB, HUMFESFPS and HUMVWFA31; HSAC04 (ACTBP2), HUMCYP19 and HUMPLA2A1; HSAC04 (ACTBP2) and HUMFABP; HUMAPOA2, HUMCYP19 and HUMPLA2A1; HUMCD4, HUMCSF1PO and HUMTH01; HUMCYP19, HUMFABP and HUMPLA2A1; HUMCYP19, HUMHPRTB and HUMPLA2A1; HUMF13A01, HUMFABP and HUMCD4; HUMHPRTB, HUMFESFPS and HUMLIPOL; HUMF13A01, HUMFABP and HUMCD4; HUMHPRTB, HUMBFXIII (F13B) and HUMPLA2A1; HUMHPRTB, HUMBFXIII (F13B) and HUMTPOX; HUMHPRTB, HUMBFXIII (F13B) and HUMFESFPS; HUMCSF1PO, HUMTPOX and HUMCD4; HUMHPRTB, HUMFESFPS and HUMMYOPK (Myotonic); HUMCSF1PO, HUMTH01 and HUMCD4; HUMCSF1PO, HUMTH01 and HUMVWFA31; HUMHPRTB, HUMBFXIII (F13B) and HUMLIPOL; HUMCSF1PO, HUMTPOX, HUMTH01 and HUM- 30 VWFA31; HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B) and HUMLIPOL; HUMCSF1PO, HUMTPOX, HUMTH01 and HUMCD4; and HUMCSF1PO, HUMTH01, HUMTPOX and HUMCD4.

The present invention provides a high throughput method 35 for the detection and analysis of polymorphic genetic markers using specific combinations of loci and specified conditions. By selection of the appropriate detection method, the process can be used in laboratories which have only a power supply and a standard apparatus for polyacrylamide gel 40 of the multiplex amplification in Example 18. electrophoresis or those which have the latest in equipment for fluorescent gel scanning, e.g., FluorImager™ -575 (Molecular Dynamics, Sunnyvale, Calif.). Thus, the process of the present invention is adaptable for a variety of uses and laboratories.

The approach as specified in the present invention produces a savings in time, labor and materials in the analysis of loci contained within the multiplexes. The process of the present invention includes all the requisite primers, allowing between two and four or more loci to be amplified together 50 in one amplification tube instead of amplifying each locus

The present invention has specific use in the field of forensic analysis, paternity determination, monitoring of bone marrow transplantation, linkage mapping, and detec- 55 of the multiplex amplification in Example 25. tion of genetic diseases and cancers.

These and other aspects of the present invention will become evident upon reference to the following detailed description of the invention and the attached drawings and photographs.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a photograph illustrating the simultaneous amplification of three loci: HUMCSF1PO, HUMTPOX and HUMTH01, with the amplified products of each locus 65 shown migrating next to the corresponding allelic ladder for ease of interpretation in Example 1.

FIG. 2 is a computer image showing the fluorescent detection of multiplex amplification of the loci HUMCSF1PO, HUMTPOX, HUMTH01 and HUM-VWFA31 as detected with a FluorImager[™] -575 (Molecular Dynamics, Sunnyvale, Calif.) in Example 2.

FIG. 3 is a photograph showing the silver stain detection of the multiplex amplification in Example 3.

FIG. 4 is a computer image showing the fluorescent detection of multiplex amplification in Example 4.

FIG. 5 is a photograph showing the silver stain detection of the multiplex amplification in Example 5.

FIG. 6 is a photograph showing the silver stain detection of the multiplex amplification in Example 6.

FIG. 7 is a photograph showing the silver stain detection of the multiplex amplification in Example 7.

FIG. 8 is a photograph showing the silver stain detection of the multiplex amplification in Example 8.

FIG. 9 is a photograph showing the silver stain detection of the multiplex amplification in Example 9.

FIG. 10 is a photograph showing the silver stain detection of the multiplex amplification in Example 10.

FIG. 11 is a photograph showing the silver stain detection of the multiplex amplification in Example 11.

FIG. 12 is a photograph showing the silver stain detection of the multiplex amplification in Example 12.

FIG. 13 is a photograph showing the silver stain detection of the multiplex amplification in Example 13.

FIG. 14 is a photograph showing the silver stain detection of the multiplex amplification in Example 14.

FIG. 15 is a photograph showing the silver stain detection of the multiplex amplification in Example 15.

FIG. 16 is a photograph showing the silver stain detection of the multiplex amplification in Example 16.

FIG. 17 is a photograph showing the silver stain detection of the multiplex amplification in Example 17.

FIG. 18 is a photograph showing the silver stain detection

FIG. 19 is a photograph showing the silver stain detection of the multiplex amplification in Example 19.

FIG. 20 is a photograph showing the silver stain detection of the multiplex amplification in Example 20.

FIG. 21 is a photograph showing the silver stain detection of the multiplex amplification in Example 21.

FIG. 22 is a photograph showing the silver stain detection of the multiplex amplification in Example 22.

FIG. 23 is a photograph showing the silver stain detection of the multiplex amplification in Example 23.

FIG. 24 is a photograph showing the silver stained detection of the multiplex amplification in example 24.

FIG. 25 is a photograph showing the silver stain detection

FIG. 26 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 26.

FIG. 27 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 27.

FIG. 28 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 28.

FIG. 29 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 29.

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FIG. 30 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 30.

FIG. 31 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in 5

FIG. 32 is a photograph of a computer image showing the fluorescent detection of the multiplex amplification in Example 32.

DETAILED DESCRIPTION OF THE INVENTION

The following definitions are intended to assist in providing a clear and consistent understanding of the scope and detail of the terms:

Allelic ladder: a standard size marker consisting of amplified alleles from the locus.

Allele: a genetic variation associated with a segment of DNA, i.e., one of two or more alternate forms of a DNA 20 sequence occupying the same locus.

Biochemical nomenclature: standard biochemical nomenclature is used herein in which the nucleotide bases are designated as adenine (A); thymine (T); guanine (G); and cytosine (C). Corresponding nucleotides are, for example, 25 deoxyguanosine-5'-triphosphate (dGTP).

DNA polymorphism: the condition in which two or more different nucleotide sequences coexist in the same interbreeding population in a DNA sequence.

Locus (or genetic locus): a specific position on a chro- 30 mosome. Alleles of a locus are located at identical sites on homologous chromosomes.

Locus-specific primer: a primer that specifically hybridizes with a portion of the stated locus or its complementary strand, at least for one allele of the locus, and does not hybridize efficiently with other DNA sequences under the conditions used in the amplification method.

Polymerase chain reaction (PCR): a technique in which cycles of denaturation, annealing with primer, and extension with DNA polymerase are used to amplify the number of copies of a target DNA sequence by >106 times. The polymerase chain reaction process for amplifying nucleic acid is covered by U. S. Pat. Nos. 4,683,195 and 4,683,202, which are incorporated herein by reference for a description of the process.

Polymorphism information content (PIC): a measure of the amount of polymorphism present at a locus (Botstein et al., 1980). PIC values range from 0 to 1.0, with higher values indicating greater degrees of polymorphism. This measure generally displays smaller values than the other commonly used measure, i.e., heterozygosity. For markers that are highly informative (heterozygosities exceeding about 70%), the difference between heterozygosity and PIC is slight.

Primary reaction: initial reaction using the purified human 55 26. HUMHPRTB, HUMFESFPS and HUMMYOPK genomic DNA as template for the PCR.

Primers: two single-stranded oligonucleotides or DNA fragments which hybridize with opposing strands of a locus such that the 3' termini of the primers are in closest proximity.

Primer pair: two primers including primer 1 that hybridizes to a single strand at one end of the DNA sequence to be amplified and primer 2 that hybridizes with the other end on the complementary strand of the DNA sequence to be amplified.

Primer site: the area of the target DNA to which a primer hybridizes.

Secondary reaction: reamplification with the same or different primer pair using a dilution of the primary reaction as template for the PCR.

Construction of the Multiplex System

Prior to constructing the multiplex system, an appropriate set of loci, primers, and amplification protocols must be selected such that amplification generates fragments such that alleles of the various loci do not overlap in size or, when such overlap occurs, fragments representing different loci are detectable by separate means. In addition, the selected loci must be compatible for use with a single amplification protocol. The specific combinations of loci described herein are unique in this application. Combinations of loci may be rejected for either of these reasons, or because, in combination, one or more of the loci do not produce adequate product yield, or fragments which do not represent authentic alleles are produced in this reaction.

Successful combinations are generated by trial and error of locus combinations and by adjustment of primer concentrations to identify an equilibrium in which all included loci may be amplified.

Of particular importance in the multiplex system is the size range of amplified alleles produced from the individual loci which will be analyzed together. For ease of analysis with current technologies, systems which can be detected by amplification of fragments smaller than 500 bases were preferably selected.

The following multiplex combinations have been developed and are considered ideal combinations for use in the present system:

- 1. HUMTH01 and HUMCSF1PO;
- 2. HUMTH01 and HUMCD4;
- 3. HUMTH01 and HUMTPOX;
- 4. HUMF13A01 and HUMFABP;
- 5. HUMF13A01 and HUMMYOPK (Myotonic);
- 6. HUMF13A01 and HUMBFXIII (F13B);
- 7. HUMBFXIII (F13B) and HUMFESFPS;
- 8. HUMBFXIII (F13B) and HUMLIPOL;
- 9. HUMHPRTB and HUMFESFPS;
- 10. HSAC04 (ACTBP2) and HUMCYP19;
- 40 11. HSAC04 (ACTBP2) and HUMFABP;
 - 12. HUMCSF1PO, HUMTPOX and HUMTH01;
 - 13. HUMHPRTB, HUMFESFPS and HUMVWFA31;
 - 14. HSAC04 (ACTBP2), HUMCYP19 and HUMPLA2A1;
- 15. HUMAPOA2, HUMCYP19 and HUMPLA2A1;
- 45 16. HUMCD4, HUMCSF1PO and HUMTH01;
 - 17. HUMCYP19, HUMFABP and HUMPLA2A1;
 - 18. HUMCYP19, HUMHPRTB and HUMPLA2A1;
 - 19. HUMF13A01, HUMFABP and HUMCD4;
 - 20. HUMHPRTB, HUMFESFPS and HUMLIPOL;
- 50 21. HUMF13A01, HUMFABP and HUMCD4;
 - 22. HUMHPRTB, HUMBFXIII (F13B) and HUMPLA2A1;
 - 23. HUMHPRTB, HUMBFXIII (F13B) and HUMTPOX;
 - 24. HUMHPRTB, HUMBFXIII (F13B) and HUMFESFPS;
 - 25. HUMCSF1PO, HUMTPOX and HUMCD4;
- (Myotonic);
 - 27. HUMCSF1PO, HUMTH01 and HUMCD4;
 - 28. HUMCSF1PO, HUMTH01 and HUMVWFA31;
 - 29. HUMHPRTB, HUMBFXIII (F13B) and HUMLIPOL;
- 60 30. HUMCSF1PO, HUMTPOX, HUMTH01 and HUM-VWFA31:
 - 31. HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B) and HUMLIPOL;
 - 32. HUMCSF1PO, HUMTPOX, HUMTH01 and HUMCD4; and
 - 33. HUMCSF1PO, HUMTH01, HUMTPOX and HUMCD4.

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The primers must also be designed so that the size of the resulting amplification products differ in length, thereby facilitating assignment of alleles to individual loci during detection. Inappropriate selection of primers can produce several undesirable effects such as lack of amplification, amplification at multiple sites, primer dimer formation, undesirable interaction of primer sequences from different loci, production of alleles from one locus which overlap with alleles from another, or the need for amplification conditions or protocols for the different loci which are incompatible in a multiplex. The synthesis of the primers is conducted by procedures known to those skilled in the art.

Using Multiplexes of Two Loci to Develop Multiplexes Using More than Two Loci

Once a multiplex containing two loci is developed, it may 15 be used as a core to create multiplexes containing more than two loci. New combinations are created including the first two loci. For example, the core multiplex containing loci HUMTH01 and HUMCSF1PO was used to generate derivative multiplexes of HUMTH01, HUMCSF1PO, and HUMT- 20 POX; HUMTH01, HUMCSF1PO, and HUMCD4;

HUMTH01, HUMCSF1PO, and HUMVWFA31; HUMTH01, HUMCSF1PO, HUMVWFA31, and HUMTPOX; and HUMTH01, HUMCSF1PO, HUMCD4, and HUMTPOX. Many other derivative multiplexes can be generated based upon a working multiplex. The derivative multiplexes are, in some sense, routine extensions of the

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Preparation of Genomic DNA

All methods of DNA preparation which are compatible with the amplification process for a single locus should be appropriate for multiplex amplification. Many examples of preparation methods have been described in the literature (Patel et al. 1984, Gill et al. 1985). DNA concentrations are measured fluorometrically (Brunk et al. 1979).

Amplification of DNA

core multiplex.

Human genomic DNA samples are subjected to PCR amplification using primers and thermocycling conditions specific for each locus. Reference is made to Table 1 for details of the primer sequences. The amplification protocol specific to each multiplex is listed in the specific examples.

Designation	Primer	se	quen	ces								-	ence umber
HSAC04	primer	1:	ACA	TCT	CCC	CTA	CCG	CTA	TA				1
(ACTBP2)	primer	2:	AAT	CTG	GGC	GAC	AAG	AGT	GA				2
HUMAPOA2	primer	1:	GGA	GCA	GTC	CTA	GGG	CCG	CGC	CGT			3
(APOCIII)	primer	2:	GTG	ACA	GAG	GGA	GAC	TCC	ATT	AAA			4
HUMCSF1PO	primer	1:	AAC	CTG	AGT	CTG	CCA	AGG	ACT	AGC			5
	primer	2:	TTC	CAC	ACA	CCA	CTG	GCC	ATC	TTC			6
HUMCYP19	primer	1:	GCA	GGT	ACT	TAG	TTA	GCT	AC				7
(CYARP450)	primer	2:	TTA	CAG	TGA	GCC	AAG	GTC	GT				8
HUMCD4	primer	1:	CCA	GGA	AGT	TGA	GGC	TGC	AGT	GAA			9
	primer	2:	TTG	GAG	TCG	CAA	GCT	GAA	CTA	GCG		1	0
HUMF13A01	primer	1:	GAG	GTT	GCA	CTC	CAG	CCT	TTG	CAA		1	1
	primer	2:	TTC	CTG	AAT	CAT	ccc	AGA	GCC	ACA		1	2
HUMBFXIII	primer	1:	TGA	GGT	GGT	GTA	CTA	CCA	TA			1	3
(F13B)	primer	2:	GAT	CAT	GCC	ATT	GCA	CTC	TA			1	4
HUMFABP	primer	1:	GTA	GTA	TCA	GTT	TCA	TAG	GGT	CAC	С	1	5
	primer	2:	CAG	TTC	GTT	TCC	ATT	GTC	TGT	CCG		1	6
HUMFESFPS	primer	1:	GCT	GTT	AAT	TCA	TGT	AGG	GAA	GGC		1	7
	primer	2:	GTA	GTC	CCA	GCT	ACT	TGG	CTA	CTC		1	8
HUMHPRTB	primer	1:	ATG	CCA	CAG	ATA	ATA	CAC	ATC	ccc		1	9
(HPRT-1)	primer	2:	CTC	TCC	AGA	ATA	GTT	AGA	TGT	AGG		2	0
HUMMYOPK	primer	1:	GCT	CGA	AGG	GTC	CTT	GTA	GCC	GGG		2	1
Myotonic	primer	2:	GAT	AGG	TGG	GGG	TGC	GTG	GAG	GAT		2	2
HUMLIPOL	primer	1:	CTG	ACC	AAG	GAT	AGT	GGG	ATA	TAG		2	3
	primer	2:	GGT	AAC	TGA	GCG	AGA	CTG	TGT	СТ		2	4
HUMPLA2A1	primer	1:	GGT	TGT	AAG	CTC	CAT	GAG	GTT	AGA		2	5

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-continued

Designation	n Primer	se	quen	ces								quence Number
(PLA-AZ)	primer	2:	TTG	AGC	ACT	TAC	TAT	GTG	CCA	GGC	T	26
HUMTH01	primer	1:	GTG	GGC	TGA	AAA	GCT	ccc	GAT	TAT		27
	primer	2:	ATT	CAA	AGG	GTA	TCT	GGG	CTC	TGG		28
HUMTPOX	primer	1:	ACT	GGC	ACA	GAA	CAG	GCA	CTT	AGG		29
	primer	2:	GGA	GGA	ACT	GGG	AAC	CAC	ACA	GGT		30
HUMVWFA31	primer	1:	GA Z	AAG (ccc :	TAG '	rgg i	ATG I	ATA I	AGA A	ATA ATC	31
	primer	2:	GGA	CAG	ATG	ATA	AAT	ACA	TAG	GAT	GGA TGG	32

details of the specific procedure relating to each multiplex. The locus-specific primers include a number of nucleotides which, under the conditions used in the hybridization, are sufficient to hybridize with an allele of the locus to be amplified and to be essentially free from amplification of alleles of other loci. Reference is made to U. S. Pat. 5,192,659 to Simons, which is incorporated herein by reference for a more detailed description of locus-specific primers.

Separation and Detection of DNA Fragments

electrophoresis, e.g., denaturing polyacrylamide gel electrophoresis (Sambrook et al., 1989). Preferred gel preparation and electrophoresis procedures are conducted as described in Example 1. Fragment separation occurs based on size and charge of the sample.

The DNA is then detected by, e.g., silver staining (Bassam et al. 1991). Alternatively, if radioactively-labeled or fluorescently-labeled primers were used for each locus, the products are detected by means available to detect these reporters as known to those skilled in the art. Amplified 40 materials may be detected using any of a number of reporters including, e.g., silver staining, radioisotopes, fluorescers, chemiluminescers and enzymes in combination with detectable substrates.

Individual DNA samples containing amplified alleles are 45 preferably compared with a size standard such as a DNA marker or locus-specific allelic ladder to determine the alleles present at each locus within the sample. The preferred size marker for evaluation of a multiplex amplification containing two or more polymorphic STR loci consists of a 50 combination of allelic ladders for the loci being evaluated.

The preferred size marker for evaluation of a multiplex amplification containing two or more polymorphic STR loci which are generated using fluorescently-labeled primers for each locus consists of a combination of fluorescently-labeled 55 allelic ladders for the loci being evaluated.

Following the construction of allelic ladders for individual loci, they may be mixed and loaded for gel electrophoresis at the same time as the loading of amplified samples occurs. Each allelic ladder co-migrates with alleles in the 60 sample from the corresponding locus.

A permanent record of the data can be generated with the use of electrophoresis duplicating film (STR systems manual #TMD004, Promega Corporation, Madison, Wis.).

Advantage of Fluorescent Detection

With the advent of automated fluorescent imaging, faster detection and analysis of multiplex amplification products

Reference is made to the examples below for additional 20 can be achieved. For fluorescent analyses, one fluoresceinated primer can be included in the amplification of each locus. Separation of the amplified fragments is achieved in precisely the same manner as with the silver stain detection method. The resulting gel is loaded onto a FluorImager® 575 (Molecular Dynamics, Sunnyvale, Calif.) which scans the gel and digitizes the data in three minutes. The FluorImager® contains an argon laser emitting 488 nm light which sweeps through the gel using a galvanometercontrolled mirror. The light activates fluorescent molecules Following amplification, products are then separated by 30 in its path and they, in turn, emit light of higher wavelength. A filter prohibits passage of the original light, but allows collection of the emitted light by a fiber optic collector. A second filter selected by the user may be inserted between the fiber optic collector and the photomultiplier, allowing detection of specific wavelength bands (or colors) with each

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The image has an overall cleaner appearance than that obtained with the silver stain for three reasons. First, only one of the two PCR product strands is labeled with primer, simplifying the two band per allele images of the silver stain. Second, in the silver stain reaction, the entire gel is exposed to silver and prone to silver deposition causing a significant general background. With the fluorescent reporter, only the primer is labeled and the unincorporated primers migrate out of the bottom of the gel prior to detection. Third, some artifact bands of the PCR reaction are plentiful, but contain very little primer.

Because this fluorescent method detects only products with one particular primer, some of these artifacts which appear in silver stain of multiplex amplifications are not detected. In fact, this characteristic has allowed development of the more complex quadriplex as shown in FIG. 2 in place of the triplex shown in FIG. 1.

The present invention is also directed to kits that utilize the process described. A basic kit includes a container having a locus-specific primer pair (or alternately separate containers containing each primer of a primer pair) for each locus. The kit also includes instructions for use.

Other ingredients may include an allelic ladder directed to each of the specified loci, a sufficient quantity of enzyme for amplification, amplification buffer to facilitate the amplification, loading solution for preparation of the amplified material for gel electrophoresis, human genomic DNA as a control to test that the system is working well, a size marker to insure that materials migrate as anticipated in the

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gel, and a protocol and manual to educate the user and to limit error in use. The amounts of the various reagents in the kits can be varied depending upon a number of factors, such as the optimum sensitivity of the process. The instructions for use are suitable to enable any analyst to carry out the 5 desired test. It is within the scope of this invention to provide test kits for use in manual applications or test kits for use with automated detectors or analyzers.

EXAMPLES

The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the disclosure or protection granted by the patent.

Genomic DNA isolation and quantitation were performed essentially as described by Puers et al., 1993. These methods are generally known to those skilled in the art and are preferred, but not required, for application of the invention.

Amplification products were separated by electrophoresis through a 0.4mm thick 4% denaturing polyacrylamide gel (19:1 ratio of acrylamide to bis-acrylamide) which contained 7 M urea (Sambrook et al., 1989) and was chemically cross-linked to one glass plate (Kobayashi, 1988). DNA samples were mixed with 3 μ l loading solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured at 95° C. for 2 min., and chilled on ice prior to loading.

Electrophoresis was performed at 60 W in 0.5× TBE for ³⁰ 1–2 hrs. The DNA was detected by silver staining (Bassam et al., 1991). Permanent images were obtained by exposure to Electrophoresis Duplicating Films (EDF, Kodak, Cat. No. 809 6232). Alternatively, detection can be performed by fluorescent scanning (Schumm et al., 1994) or radioactive ³⁵ detection (Hammond et al., 1994).

Example 1

Silver Stain Detection of Multiplex Amplification of Loci HUMCSF1PO, HUMTPOX, and HUMTH01

In this example, a DNA template (three DNA samples) was amplified at the individual loci HUMCSF1PO, HUMTPOX, and HUMTH01 simultaneously in a single reaction vessel. The PCR amplifications were performed in 50 μ l volumes using 25 ng template, 0.03 U Taq DNA Polymerase/ μ l, 1×STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl2 and 200 μ M each of dATP, dCTP, dGTP and dTTP), and using a Thermal Cycler 480 (Perkin Elmer Cetus). Amplification protocol 1 (96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 64° C. for 1 min., followed by 20 cycles of 90° C. for 1 min., 64° C. for 1 min., 55° C. for 1.5 min.) was employed.

Six amplification primers were used in combination, including 0.2 μ M each HUMCSF1PO primers 1 [SEQ. ID. 5] and 2 [SEQ. ID. 6], 0.2 μ M each HUMTPOX primers 1 [SEQ. ID. 29] and 2 [SEQ. ID. 30], and 0.6 μ M each HUMTH01 primers 1 [SEQ. ID. 27] and 2 [SEQ. ID. 28].

Amplified products were separated by denaturing acrylamide gel electrophoresis on a 40 cm gel for 60–90 min. at 60 W and products were visualized by silver stain analysis according the protocol of *Bassam* et al. (1991).

Reference is made to FIG. 1 which reveals the silver stain detection of the multiplex amplification. Lanes 2, 3, and 5

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contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO, HUMTPOX, and HUMTH01. Lanes 1, 4, and 7 contain allelic ladders for the three loci and lane 6 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

Example 2

Fluorescent Detection of Multiplex Amplification of Loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMVWFA31

In this example, a DNA template was amplified at the individual loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMVWFA31 simultaneously in a single reaction vessel. The PCR amplifications were performed in 25 μ l volumes using 25 ng template, 0.04 U Taq DNA Polymerase/µl, 1×STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl2 and 200 µM each of DATP, dCTP, dGTP and dTTP), and using a Thermal Cycler 480 (Perkin Elmer Cetus). Amplification protocol 1, as described in Example 1, was employed. Eight amplification primers were used in combination, including 1 μ M each HUMCSF1PO primer 2 [SEQ. ID 6] and fluorescein-labeled primer 1 [SEQ. ID. 5], $0.15 \mu M$ each HUMTPOX primer 1 [SEQ. ID. 29] and fluorescein-labeled primer 2 [SEQ. ID. 30], 0.2 µM each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27], and 1 μ M each HUMVWFA31 primer 1 [SEQ. ID. 31] and fluorescein-labeled primer 2 [SEQ. ID. 32].

Amplified products were separated by denaturing acrylamide gel electrophoresis on a 32 cm gel for 45 minutes at 40 watts. Detection of the fluorescent signal was achieved using the FluorImager™ 575 (Molecular Dynamics, Sunnyvale, Calif.). Reference is made to FIG. 2 which is a computer image of a FluorImager scan. Lanes 2–7 contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMVWFA31. Lane 1 contains allelic ladders for the 4 loci.

Example 3

Multiplex Amplification of Loci HUMHPRTB, HUMFESFPS, and HUMVWFA31

In this example, a DNA template was amplified at the loci HUMHPRTB, HUMFESFPS, and HUMVWFA31 simultaneously in a single reaction vessel. The PCR amplifications were performed in 25 μ l volumes using 25 ng template, 0.03 U Taq DNA Polymerase/μl, 1×STR Buffer (described in example 1), and a Thermal Cycler 480 (Perkin Elmer Cetus). Amplification protocol 2 (96° C. for 2 min., then 10 cycles of 94° C. for 1 min., 60° C. for 1 min., and 70° C. for 1.5 min., followed by 20 cycles of 90° C. for 1 min., 64° C. for 1 min., 70° C. for 1.5 min.) was employed. Amplified products were separated by denaturing acrylamide gel electrophoresis on a 32 cm gel for 45 min. at 40 W and products were visualized by silver stain analysis according the protocol of Bassam et al. (supra.). Six primers were used in combination including $0.2 \mu M$ each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], 1.5 μ M each HUM-FESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18], and 1 μ M each HUMVWFA31 primers 1 [SEQ. ID. 31] and 2 [SEQ. ID. 32].

Reference is made to FIG. 3 which reveals the silver stain detection of the multiplex amplification. Lanes 2–6 contain DNA samples simultaneously co-amplified for the loci

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HUMHPRTB, HUMFESFPS, and HUMVWFA31. Lanes 1 and 7 contain allelic ladders for the 3 loci.

Example 4

Fluorescent Detection of Multiplex Amplification of Loci HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B), and HUMLIPOL

In this example, a DNA template was amplified at the loci HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B), and 10 HUMLIPOL simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 2 using amplification protocol 2, as described in Example 3.

Eight primers were used in combination, including 1 μ M 15 each HUMHPRTB primer 2 [SEQ. ID. 20] and fluoresceinlabeled primer 1 [SEQ. ID.19], 2.5 μ M each HUMFESFPS primer 2 [SEQ. ID. 18] and fluorescein-labeled primer 1 [SEQ. ID. 17], 1 μ M each HUMBFXIII (F13B) primer 2 [SEQ. ID. 14] and fluorescein-labeled primer 1 [SEQ. ID. 13], and 0.5 μ M each HUMLIPOL primer 2 [SEQ. ID. 24] and fluorescein-labeled primer 1 [SEQ. ID. 23].

Reference is made to FIG. 4 which is a computer image of a FluorImager scan. Lanes 2-7 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMFESFPS, HUMBFXIII (F13B), and HUMLIPOL. Lane 1 contains allelic ladders for the 4 loci.

Example 5

Multiplex Amplification of Loci HSAC04 (ACTBP2) and HUMCYP19

In this example, a DNA template was amplified at the individual loci HSAC04 and HUMCYP19 simultaneously in 35 a single reaction vessel. The PCR amplifications were performed in 15 μ l volumes with 25 ng template, 0.01 U Taq DNA Polymerase/µl, 1×Taq DNA Polymerase Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100 and 1.5 mM MgCl2) and 200 μ M each of dATP, 40 dCTP, dGTP and dTTP using a Thermal Cycler 480 (Perkin Elmer Cetus). Amplification protocol 2, as described in Example 3, was employed. Amplified products were separated and detected per example 1. Four primers were used in combination, including 1 µM each HSAC04 (ACTBP2) 45 Example 3. Six primers were used in combination, including primers 1 [SEQ. ID. 1] and 2 [SEQ. ID. 2], and 1 μ M each HUMCYP19 primers 1 [SEQ. ID. 7] and 2 [SEQ. ID. 8].

Reference is made to FIG. 5 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain HSAC04 (ACTBP2) and HUMCYP19. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

Example 6

Multiplex Amplification of Loci HSAC04 (ACTBP2), HUMCYP19, and HUMPLA2A1

In this example, a DNA template was amplified at the loci HSAC04 (ACTBP2), HUMCYP19, and HUMPLA2A1 60 simultaneously in a single reaction vessel. The PCR amplifications were performed in 15 μ l volumes with 25 ng template, 0.02 U Taq DNA Polymerase/µl, 1×Taq DNA Polymerase Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton X-100 and 1.5 mM MgCl2) and 200 65 µM each of DATP, dCTP, dGTP and dTTP using a Thermal Cycler 480 (Perkin Elmer Cetus). Amplification protocol 2,

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as described in Example 3, was employed. Amplified products were separated and detected per example 1. Six primers were used in combination, including 1 µM each HSAC04 (ACTBP2) primers 1 [SEQ. ID. 1] and 2 [SEQ. ID. 2], $1 \mu M$ each HUMPLA2A1 primers 1 [SEQ. ID. 25] and 2 [SEQ. ID. 26], and 1 μ M each HUMCYP19 primers 1 [SEQ. ID. 7] and 2 [SEQ. ID. 8].

Reference is made to FIG. 6 which reveals the silver stain detection of the multiplex amplification. Lanes 1–3 contain DNA samples simultaneously co-amplified for the loci HSAC04 (ACTBP2), HUMCYP19, and HUMPLA2A1. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

Example 7

Multiplex Amplification of Loci HSAC04 (ACTBP2) and HUMFABP

In this example, a DNA template was amplified at the loci HSAC04 (ACTBP2) and HUMFABP simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 5 using amplification protocol 2, as described in Example 3. Four primers were used in combination, 1 μ M each HSAC04 (ACTBP2) primers 1 [SEQ. ID. 1] and 2 [SEQ. ID. 2], and 1 μM each HUMFABP primers 1 [SEQ. ID. 15] and 2 [SEQ.

Reference is made to FIG. 7 which reveals the silver stain detection of the multiplex amplification. Lanes 1–3 contain DNA samples simultaneously co-amplified for the loci HSAC04 (ACTBP2) and HUMFABP. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

Example 8

Multiplex Amplification of Loci HUMAPOA2, HUMCYP19, and HUMPLA2A1

In this example, a DNA template was amplified at the loci HUMAPOA2, HUMCYP19, and HUMPLA2A simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 6 using amplification protocol 2, as described in 1 μ M each HUMAPOA2 primers 1 [SEQ. ID. 3] and 2 [SEQ. ID. 4], 1 μ M each HUMCYP19 primers 1 [SEQ. ID. 7] and 2 [SEQ. ID. 8], and 1 μ M each HUMPLA2A1 primers 1 [SEQ. ID. 25] and 2 [SEQ. ID. 26]. Reference is made to DNA samples simultaneously co-amplified for the loci 50 FIG. 8 which reveals the silver stain detection of the multiplex amplification. Lanes 1 and 3 contain DNA samples simultaneously co-amplified for the loci HUMAPOA2, HUMCYP19, and HUMPLA2A1. Lane 2 contains a DNA sample which failed to amplify and lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

Example 9

Multiplex Amplification of Loci HUMCD4, HUMCSF1PO, and HUMTH01

In this example, a DNA template was amplified at the loci HUMCD4, HUMCSF1PO, and HUMTH01 simultaneously in a single reaction vessel. The PCR amplifications were performed in 50 μ l volumes with 25 ng template, 0.02 U Taq DNA Polymerase/μl, 1×Taq DNA Polymerase Buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25° C.), 0.1% Triton

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X-100 and 1.5 mM MgCl2) and 200 μ M each of DATP, dCTP, dGTP and dTTP using a Thermal Cycler 480 (Perkin Elmer Cetus). Amplification protocol 1, as described in Example 1, was employed. Amplified products were separated and detected as described in Example 1. Six primers 5 were used in combination, including 1 μM each HUMCD4 primers 1 [SEQ. ID. 9] and 2 [SEQ. ID. 10], 1 μ M each HUMCSF1PO primers 1 [SEQ. ID. 5] and 2 [SEQ. ID. 6], and 1 μM each HUMTH01 primers 1 [SEQ. ID. 27] and 2 [SEQ. ID. 28].

Reference is made to FIG. 9 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMCD4, HUMCSF1PO, and HUMTH01. Lane 4 displays a sample without DNA subjected to the same procedures, 15 i.e., a negative control.

Example 10

Multiplex Amplification of Loci HUMCYP19, HUMFABP, and HUMPLA2A1

In this example, a DNA template was amplified at the loci HUMCYP19, HUMFABP, and HUMPLA2A1 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 6 using amplification protocol 2, as described in Example 3. Six primers were used in combination, including 1 μM each HUMCYP19 primers 1 [SEQ. ID. 7] and 2 [SEQ. ID. 8], 1 µM each HUMFABP primers 1 [SEQ. ID. 15] and 2 [SEQ. ID. 16] and 1 μM each HUMPLA2A1 primers 1 [SEQ. ID. 25] and 2 [SEQ. ID. 26].

Reference is made to FIG. 10 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMCYP19, HUMFABP, and HUMPLA2A1. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

Example 11

Multiplex Amplification of Loci HUMCYP19, HUMHPRTB, and HUMPLA2A1

In this example, a DNA template was amplified at the loci HUMCYP19, HUMHPRTB, and HUMPLA2A1 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 9 using amplification protocol 2, as described in Example 3. Six primers were used in combination, including $1 \,\mu\text{M}$ each HUMCYP19 primers 1 [SEQ. ID. 7] and 2 [SEQ. ID. 8], 1 μ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], and 1 μ M each HUMPLA2A1 primers 1 [SEQ. ID. 25] and 2 [SEQ. ID. 26].

Reference is made to FIG. 11 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 55 subjected to the same procedures, i.e., a negative control. contain DNA samples simultaneously co-amplified for the loci HUMCYP19, HUMHPRTB, and HUMPLA2A1. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

Example 12

Multiplex Amplification of Loci HUMF13A01 and HUMFABP

In this example, a DNA template was amplified at the loci 65 HUMF13A01 and HUMFABP simultaneously in a single reaction vessel. The PCR amplifications and other manipu16

lations were performed as described in Example 5 using amplification protocol 1, as described in Example 1. Four primers were used in combination, including 1 µM each HUMF13A01 primers 1 [SEQ. ID. 11] and 2 [SEQ. ID. 12], and 1 μ M each HUMFABP primers 1 [SEQ. ID. 15] and 2 [SEQ. ID. 16].

Reference is made to FIG. 12 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMF13A01 and HUMFABP. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

Example 13

Multiplex Amplification of Loci HUMBFXIII (F13B) and HUMFESFPS

In this example, a DNA template was amplified at the loci HUMBFXIII (F13B) and HUMFESFPS simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 6 using amplification protocol 1, as described in Example 1. Four primers were used in combination, including 1 μ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14], and 1 μ M each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18].

Reference is made to FIG. 13 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMBFXIII (F13B) and HUMFESFPS. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

Example 14

Multiplex Amplification of Loci HUMBFXIII (F13B), HUMHPRTB, and HUMPLA2A1

In this example, a DNA template was amplified at the loci 40 HUMBFXIII (F13B), HUMHPRTB, and HUMPLA2A1 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 6 using amplification protocol 2, as described in Example 3. Six primers were used in combination, including 1 µM each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14], 1 μ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], and 1 µM each HUMPLA2A1 primers 1 [SEQ. ID. 25] and 2 [SEQ. ID. 26].

Reference is made to FIG. 14 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMBFXIII (F13B), HUMHPRTB, and HUMPLA2A1. Lane 4 displays a sample without DNA

Example 15

Multiplex Amplification of Loci HUMF13A01, HUMFABP, and HUMCD4

In this example, a DNA template was amplified at the loci HUMF13A01, HUMFABP, and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 5 using amplification protocol 1, as described in Example 1. Six primers were used in combination, including 1 μ M each HUMF13A01 primers 1 [SEQ. ID. 11] and 2 [SEQ. ID. 12],

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1 μ M each HUMFABP primers 1 [SEQ. ID. 15] and 2 [SEQ. ID. 16], and 1 μ M each HUMCD4 primers 1 [SEQ. ID. 9] and 2 [SEQ. ID. 10].

Reference is made to FIG. 15 which reveals the silver stain detection of the multiplex amplification. Lanes 1–3 ⁵ contain DNA samples simultaneously co-amplified for the loci HUMF13A01, HUMFABP, and HUMCD4. Lane 4 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

Example 16

Multiplex Amplification of Loci HUMHPRTB and HUMFESFPS

In this example, a DNA template was amplified at the loci 15 HUMHPRTB and HUMFESFPS simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using $^{500-0.5}$ ng template, $^{0.02}$ U Taq DNA Polymerase/ $^{\mu}$ l and amplification protocol 2, as described in Example 3. Four 20 primers were used in combination, including $^{0.2}$ $^{\mu}$ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20] and $^{1.5}$ $^{\mu}$ M each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18].

Reference is made to FIG. **16** which reveals the silver ²⁵ stain detection of the multiplex amplification. Lanes 1–6 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB and HUMFESFPS using 500, 50, 25, 5, 1 and 0.5 ng DNA template. Lane 7 displays a sample without DNA subjected to the same procedures, i.e., a negative ³⁰ control.

Example 17

Multiplex Amplification of Loci HUMHPRTB, HUMFESFPS, and HUMLIPOL

In this example, a DNA template was amplified at the loci HUMHPRTB, HUMFESFPS, and HUMLIPOL simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using amplification protocol 2, as described in Example 3. Six primers were used in combination, including 0.4 μ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], 3 μ M each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18], and 2 μ M each HUMLIPOL 45 primers 1 [SEQ. ID. 23] and 2 [SEQ. ID. 24].

Reference is made to FIG. 17 which reveals the silver stain detection of the multiplex amplification. Lanes 1–3 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMFESFPS and HUMLIPOL. Lane 4 50 displays a sample without DNA subjected to the same procedures, i.e., a negative control.

Example 18

Multiplex Amplification of Loci HUMBFXIII (F13B) and HUMLIPOL

In this example, a DNA template was amplified at the loci HUMBFXIII (F13B) and HUMLIPOL Simultaneously in a single reaction vessel. The PCR amplifications and other 60 manipulations were performed as described in Example 1 using 0.02 U Taq DNA Polymerase/ μ l and amplification protocol 2, as described in Example 3. Four primers were used in combination, including 1 μ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14] and 1 65 μ M each HUMLIPOL primers 1 [SEQ. ID. 23] and 2 [SEQ. ID. 24].

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Reference is made to FIG. 18 which reveals the silver stain detection of the multiplex amplification. Lanes 1–3 contain DNA samples simultaneously co-amplified for the loci HUMBFXIII (F13B) and HUMLIPOL.

Example 19

Multiplex Amplification of Loci HUMHPRTB, HUMTPOX, and HUMBFXIII (F13B)

In this example, a DNA template was amplified at the loci HUMHPRTB, HUMTPOX, and HUMBFXIII (F13B) simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using amplification protocol 2, as described in Example 3. Six primers were used in combination, including 1 μM each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], 0.2 μM each HUMTPOX primers 1 [SEQ. ID. 29] and 2 [SEQ. ID. 30], and 2 μM each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14].

Reference is made to FIG. 19 which reveals the silver stain detection of the multiplex amplification. Lanes 1–3 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMTPOX, and HUMBFXIII (F13B).

Example 20

Multiplex Amplification of Loci HUMHPRTB, HUMFESFPS, and HUMBFXIII (F13B)

In this example, a DNA template was amplified at the loci HUMHPRTB, HUMFESFPS, and HUMBFXIII (F13B) simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using amplification protocol 2, as described in Example 3. Six primers were used in combination, including 1 μ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 [SEQ. ID. 20], 2 μ M each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18], and 2 μ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14].

Reference is made to FIG. 20 which reveals the silver stain detection of the multiplex amplification. Lanes 1–3 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMFESFPS, and HUMBFXIII (F13B).

Example 21

Multiplex Amplification of Loci HUMCSF1PO, HUMTPOX, and HUMCD4

In this example, a DNA template was amplified at the loci HUMCSF1PO, HUMTPOX, and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using amplification protocol 1, as described in Example 1. Six primers were used in combination, including 1 μ M each HUMCSF1PO primers 1 [SEQ. ID. 5] and 2 [SEQ. ID. 6], 1 μ M each HUMTPOX primers 1 [SEQ. ID. 29] and 2 [SEQ. ID. 30], and 1 μ M each HUMCD4 primers 1 [SEQ. ID. 9] and 2 [SEQ. ID. 10].

Reference is made to FIG. 21 which reveals the silver stain detection of the multiplex amplification. Lanes 1–3 contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO, HUMTPOX, and HUMCD4.

Example 22

Multiplex Amplification of Loci HUMHPRTB, HUMFESFPS, and HUMMYOPK (Myotonic)

In this example, a DNA template was amplified at the loci HUMHPRTB, HUMFESFPS, and HUMMYOPK simulta-

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neously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using amplification protocol 2, as described in Example 3. Six primers were used in combination, including 1 μ M each HUMHPRTB primers 1 [SEQ. ID. 19] and 2 5 [SEQ. ID. 20], 1 μ M each HUMFESFPS primers 1 [SEQ. ID. 17] and 2 [SEQ. ID. 18], and 1 μ M each HUMMYOPK (Myotonic) primers 1 [SEQ. ID. 21] and 2 [SEQ. ID. 22].

Reference is made to FIG. 22 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 10 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMFESFPS, and HUMMYOPK (Myotonic).

Example 23

Multiplex Amplification of Loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4

In this example, a DNA template was amplified at the loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 using 0.04 U Taq DNA Polymerase/ μ l and amplification protocol 1, as described in Example 1. Eight primers were used in combination, including 1 μ M each HUMCSF1PO primers 1 [SEQ. ID. 5] and 2 [SEQ. ID. 6], 1 μ M each HUMTPOX primers 1 [SEQ. ID. 29] and 2 [SEQ. ID. 30], 1 µM each HUMTH01 primers 1 [SEQ. ID. 27] and 2 [SEQ. ID. 28], and 1 μ M each HUMCD4 primers 1 [SEQ. ID. 9] and 2 [SEQ. ID. 10].

Reference is made to FIG. 23 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO, HUMTPOX, HUMTH01, and 35 HUMCD4.

Example 24

Multiplex Amplification of Loci HUMF13A01 and HUMMYOPK (Myotonic)

In this example, a DNA template was amplified at the loci HUMF13A01 and HUMMYOPK (Myotonic) simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in 45 Example 1 using 0.04 U Taq DNA Polymerase/µl and amplification protocol 1, as described in Example 1. Four primers were used in combination, including 0.1 µM each HUMF13A01 primers 1 [SEQ. ID. 11] and 2 [SEQ. ID. 12] and 1 μ M each HUMMYOPK (Myotonic) primers 1 [SEQ. 50 labeled primer 2 [SEQ. ID. 10]. ID. 21] and 2 [SEQ. ID. 22].

Reference is made to FIG. 24 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMF13A01 and HUMMYOPK (Myotonic).

Example 25

Multiplex Amplification of Loci HUMF13A01 and HUMBFXIII (F13B)

In this example, a DNA template was amplified at the loci HUMF13A01 and HUMBFXIII (F13B) simultaneously in a single reaction vessel. The PCR amplifications and other manipulations were performed as described in Example 1 used in combination, including 0.1 µM each HUMF13A01

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primers 1 [SEQ. ID. 11] and 2 [SEQ. ID. 12] and 0.5 μ M each HUMBFXIII (F13B) primers 1 [SEQ. ID. 13] and 2 [SEQ. ID. 14].

Reference is made to FIG. 25 which reveals the silver stain detection of the multiplex amplification. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMF13A01 and HUMBFXIII (F13B).

Example 26

Fluorescent Detection of Multiplex Amplification of Loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4

In this example, a DNA template was amplified at the 15 individual loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.04 U Taq DNA Polymerase/µl and amplification protocol 1, as described in Example 1. Eight amplification primers were used in combination, including 2 μM each HUMCSF1PO primer 2 [SEQ. ID. 6] and fluorescein-labeled primer 1 [SEQ. ID. 5], 0.5 µM each HUMTPOX primer 1 [SEQ. ID. 29] and fluorescein-labeled primer 2 [SEQ. ID. 30], 0.5 μ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27] and 0.5 μ M each HUMCD4 primer 1 [SEQ. ID. 9] and fluorescein-labeled primer 2 [SEQ. ID. 10].

Amplified products were detected as in Example 2. Reference is made to FIG. 26 which is a photograph of a computer image of a FluorImager scan. Lanes 1-3 contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO, HUMTPOX, HUMTH01, and HUMCD4.

Example 27

Fluorescent Detection of Multiplex Amplification of Loci HUMCSF1PO, HUMTH01, and HUMCD4

In this example, a DNA template was amplified at the $_{40}$ individual loci HUMCSF1PO, $\overline{\text{HUMTH01}}$, and $\overline{\text{HUMCD4}}$ simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.02 U Taq DNA Polymerase/µl and amplification protocol 1, as described in Example 1. Six amplification primers were used in combination, including 1 µM each HUMCSF1PO primer 2 [SEQ. ID. 6] and fluorescein-labeled primer 1 [SEQ. ID. 5], 1 μ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27] and 1 μ M each HUMCD4 primer 1 [SEQ. ID. 9] and fluorescein-

Amplified products were detected as in Example 2. Reference is made to FIG. 27 which is a photograph of a computer image of a FluorImager scan. Lanes 1 and 2 contain DNA samples simultaneously co-amplified for the 55 loci HUMCSF1PO, HUMTH01, and HUMCD4.

Example 28

Fluorescent Detection of Multiplex Amplification of Loci HUMCSF1PO, HUMTH01, and **HUMVWFA31**

In this example, a DNA template was amplified at the individual loci HUMCSF1PO, HUMTH01, and HUM-VWFA31 simultaneously in a single reaction vessel. The using 0.03 U Taq DNA Polymerase/µl and amplification 65 PCR amplifications were performed as described in protocol 2, as described in Example 3. Four primers were Example 1 using 0.02 U Taq DNA Polymerase/µl and amplification protocol 1, as described in Example 1. Six

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amplification primers were used in combination, including 1 μ M each HUMCSF1PO primer 2 [SEQ. ID. 6] and fluorescein-labeled primer 1 [SEQ. ID. 5], 1 μ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27], and 1 μ M each HUMVWFA31 5 primer 1 [SEQ. ID. 31] and fluorescein-labeled primer 2 [SEQ. ID. 32].

Amplified products were detected as in Example 2. Reference is made to FIG. **28** which is a photograph of a computer image of a FluorImager scan. Lanes 1 and 2 to DNA samples simultaneously contain DNA samples simultaneously co-amplified for the loci HUMCSF1PO, HUMTH01, and HUMVWFA31.

Example 29

Fluorescent Detection of Multiplex Amplification of Loci HUMHPRTB, HUMBFXIII (F13B), and HUMLIPOL

In this example, a DNA template was amplified at the individual loci HUMHPRTB, HUMBFXIII (F13B), and HUMLIPOL simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.03 U Taq DNA Polymerase/ μ l and amplification protocol 2, as described in Example 3. Six amplification primers were used in combination, including 1 μ M each HUMHPRTB primer 2 [SEQ. ID. 20] and fluorescein-labeled primer 1 [SEQ. ID. 19], 1 μ M each HUMBFXIII (F13B) primer 2 [SEQ. ID. 14] and fluorescein-labeled primer 1 [SEQ. ID. 13], and 1 μ M each HUMLIPOL primer 2 [SEQ. ID. 24] and fluorescein-labeled primer 1 [SEQ. ID. 23].

Amplified products were detected as in Example 2. Reference is made to FIG. 29 which is a photograph of a computer image of a FluorImager scan. Lanes 1–3 contain DNA samples simultaneously co-amplified for the loci HUMHPRTB, HUMBFXIII (F13B), and HUMLIPOL.

Example 30

Fluorescent Detection of Multiplex Amplification of Loci HUMCSF1PO and HUMTH01

In this example, a DNA template was amplified at the individual loci HUMCSF1PO and HUMTH01 simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.02 U Taq DNA Polymerase/ μ l and amplification protocol 1, as described in Example 1. Four amplification primers were used in combination, including 2 μ M each HUMCSF1PO primer 2 [SEQ. ID. 6] and fluorescein-labeled primer 1 [SEQ. ID. 5] and 1 μ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27].

Amplified products were detected as in Example 2. Reference is made to FIG. **30** which is a photograph of a computer image of a FluorImager scan. Lanes 1–3 contain 55 DNA samples simultaneously co-amplified for the loci HUMCSF1PO and HUMTH01.

Example 31

Fluorescent Detection of Multiplex Amplification of Loci HUMTH01 and HUMCD4

In this example, a DNA template was amplified at the individual loci HUMTH01 and HUMCD4 simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.02 U Taq DNA Polymerase/µl and amplification protocol 1, as described in prenatal diag

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Example 1. Four amplification primers were used in combination, including 1 μ M each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27] and 1 μ M each HUMCD4 primer 1 [SEQ. ID. 9] and fluorescein-labeled primer 2 [SEQ. ID. 10].

Amplified products were detected as in Example 2. Reference is made to FIG. 31 which is a photograph of a computer image of a FluorImager scan. Lanes 1–3 contain DNA samples simultaneously co-amplified for the loci HUMTH01 and HUMCD4.

Example 32

Fluorescent Detection of Multiplex Amplification of Loci HUMTH01 and HUMTPOX

In this example, a DNA template was amplified at the individual loci HUMTH01 and HUMTPOX simultaneously in a single reaction vessel. The PCR amplifications were performed as described in Example 1 using 0.02 U Taq DNA Polymerase/µl and amplification protocol 1, as described in Example 1. Four amplification primers were used in combination, including 1 µM each HUMTH01 primer 2 [SEQ. ID. 28] and fluorescein-labeled primer 1 [SEQ. ID. 27] and 1 µM each HUMTPOX primer 2 [SEQ. ID. 30] and fluorescein-labeled primer 1 [SEQ. ID. 29].

Amplified products were detected as in Example 2. Reference is made to FIG. **32** which is a photograph of a computer image of a FluorImager scan. Lanes 1–3 contain DNA samples simultaneously co-amplified for the loci HUMTH01 and HUMTPOX.

It is understood that the invention is not confined to the particular construction and arrangements herein illustrated and described, but embraces such modified forms thereof and come within the scope of the claims following the bibliography.

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SEQUENCE LISTING

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(iii) NUMBER OF SEQUENCES: 32

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(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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	(ii) MOLECULE TYPE: DNA (ge	enomic)		
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	(ii) MOLECULE TYPE: DNA (ge	enomic)		
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	(i) SEQUENCE CHARACTERIST	CCS:		

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	(A) LENGTH: 20 base pairs(B) TYPE: nucleic acid(C) STRANDEDNESS: single(D) TOPOLOGY: linear		
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(ii)	MOLECULE TYPE: DNA (genomic)		
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(i)	(A) LENGTH: 24 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear		

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:	

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-continued GCTGTTAATT CATGTAGGGA AGGC 24 (2) INFORMATION FOR SEQ ID NO: 18: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18: GTAGTCCCAG CTACTTGGCT ACTC 24 (2) INFORMATION FOR SEQ ID NO: 19: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19: ATGCCACAGA TAATACACAT CCCC (2) INFORMATION FOR SEQ ID NO: 20: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20: CTCTCCAGAA TAGTTAGATG TAGG 24 (2) INFORMATION FOR SEQ ID NO: 21: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21: GCTCGAAGGG TCCTTGTAGC CGGG (2) INFORMATION FOR SEQ ID NO: 22: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22: GATAGGTGGG GGTGCGTGGA GGAT 2.4

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(2) INFORMATION FOR SEQ ID NO: 23:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID	No: 23:
CTGACCAAGG ATAGTGGGAT ATAG	24
(2) INFORMATION FOR SEQ ID NO: 24:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 23 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID	NO: 24:
GGTAACTGAG CGAGACTGTG TCT	23
(2) INFORMATION FOR SEQ ID NO: 25:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID	No: 25:
GGTTGTAAGC TCCATGAGGT TAGA	24
(2) INFORMATION FOR SEQ ID NO: 26:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 25 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID	NO: 26:
TTGAGCACTT ACTATGTGCC AGGCT	25
(2) INFORMATION FOR SEQ ID NO: 27:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID	NO: 27:
GTGGGCTGAA AAGCTCCCGA TTAT	24
(2) INFORMATION FOR SEQ ID NO: 28:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 24 base pairs	

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-continued

(B	TYPE:	nucleic	acid
(D) libra	HUCTETO	acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

ATTCAAAGGG TATCTGGGCT CTGG

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(2) INFORMATION FOR SEQ ID NO: 29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

ACTGGCACAG AACAGGCACT TAGG

2.4

- (2) INFORMATION FOR SEQ ID NO: 30:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GGAGGAACTG GGAACCACAC AGGT

24

- (2) INFORMATION FOR SEQ ID NO: 31:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GAAAGCCCTA GTGGATGATA AGAATAATC

29

- (2) INFORMATION FOR SEQ ID NO: 32:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GGACAGATGA TAAATACATA GGATGGATGG

What is claimed is:

1. A method of simultaneously determining the alleles present in at least three short tandem repeat loci from one or more DNA samples, comprising:

a) obtaining at least one DNA sample to be analyzed;b) selecting a set of at least three short tandem repeat loci of the DNA sample to be analyzed which can be

co-amplified, wherein the at least three short tandem repeat loci in the set comprises at least three loci selected from the group consisting of:

HUMPOX, HUMTH01 and HUMCD4; HUMTPOX, HUMTH01 and HUMVWFA31; HUMHPRTB, HUMFESFPS and HUMVWFA31;

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HSAC04 (ACTBP2), HUMCYP19 and HUMPLA2A1;

HUMAPOA2, HUMCYP19 and HUMPLA2A1; HUMCD4, HUMCSF1PO and HUMTH01;

HUMCYP19, HUMFABP and HUMPLA2A1; HUMCYP19, HUMHPRTB and HUMPLA2A1;

HUMHPRTB, HUMFESFPS and HUMLIPOL; HUMF13AO1, HUMFABP and HUMCD4;

HUMHPRTB, HUMBFXIII (F13B) and HUMPLA2A1;

HUMHPRTB, HUMBFXIII (F13B) and HUMTPOX; HUMHPRTB, HUMBFXIII (F13B) and HUMFES-FPS.

HUMCSF1PO, HUMTPOX and HUMCD4; HUMHPRTB, HUMFESFPS and HUMMYOPK 15

(Myotonic); HUMCSF1PO, HUMTH01 and HUMCD4; HUMCSF1PO, HUMTH01 and HUMVWFA31; and HUMHPRTB, HUMBFXIII (F13B) and HUMLIPOL;

- c) co-amplifying the set of at least three short tandem ²⁰ repeat loci in a multiplex amplification reaction, thereby producing a mixture of amplified alleles from each of the co-amplified loci in the set; and
- d) evaluating the amplified alleles in the mixture to determine the alleles present at each of the co-amplified ²⁵ loci in the set.
- 2. The method of claim 1 wherein in step (b), the at least three loci are co-amplified by multiplex polymerase chain reaction.
- 3. The method of claim 1 wherein the at least three loci are co-amplified using at least one oligonucleotide primer pair consisting of two oligonucleotide primers, at least one of which has a sequence selected from a group of sequences consisting of:

SEQ ID. NO. 1 and SEQ ID. NO. 2 when one of the loci in the set is HSAC04;

SEQ ID. NO. 3 and SEQ ID. NO. 4 when one of the loci in the set is HUMAPOA2;

SEQ ID. NO. 5 and SEQ ID. NO. 6 when one of the loci 40 in the set is HUMCSF1PO;

SEQ ID. NO. 7 and SEQ ID. NO. 8 when one of the loci in the set is HUMCYP19;

SEQ ID. NO. 9 and SEQ ID. NO. 10 when one of the loci in the set is HUMCD4;

SEQ ID. NO. 11 and SEQ ID. NO. 12 when one of the loci in the set is HUMF13A01;

SEQ ID. NO.13 and SEQ ID. NO. 14 when one of the loci in the set is HUMBFXIII;

SEQ ID. NO. 15 and SEQ ID. NO. 16 when one of the loci in the set is HUMFABP;

SEQ ID. NO. 17 and SEQ ID. NO. 18 when one of the loci in the set is HUMFESFPS;

SEQ ID. NO. 19 and SEQ ID. NO. 20 when one of the loci 55 in the set is HUMHPRTB;

SEQ ID. NO. 21 and SEQ ID. NO. 22 when one of the loci in the set is HUMMYOPK (Myotonic);

SEQ ID. NO. 23 and SEQ ID. NO. 24 when one of the loci in the set is HUMLIPOL;

SEQ ID. NO. 25 and SEQ ID. NO. 26 when one of the loci in the set is HUMPLA2A1;

SEQ ID. NO. 27 and SEQ ID. NO. 28 when one of the loci in the set is HUMTH01;

SEQ ID. NO. 29 and SEQ ID. NO. 30 when one of the loci in the set is HUMTPOX; and

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SEQ ID. NO. 31 and SEQ ID. NO. 32 when one of the loci in the set is HUMVWFA31.

- 4. The method of claim 1, wherein the amplified alleles are evaluated in step (d) by separating the alleles and comparing the separated alleles to a size standard selected from a DNA size marker or a locus-specific allelic ladder.
- 5. The method of claim 1, further comprising the step of separating the alleles by denaturing polyacrylamide gel electrophoresis.
- 6. The method of claim 5 wherein the separated alleles are detected by silver staining.
- 7. The method of claim 5 wherein the separated alleles are detected by fluorescence detection.
 - 8. The method of claim 1, further comprising:

identifying primers for co-amplifying each locus in the set of loci selected in step (b) such that the amplified alleles produced in the multiplex amplification reaction of step (c) do not overlap when separated to evaluate the amplified alleles in step (e); and

using the primers in the multiplex amplification reaction in step (c).

- 9. The method of claim 1 wherein the at least one DNA sample to be analyzed is selected from the group consisting of blood, semen, vaginal cells, hair, saliva, urine or other tissue, placental cells or fetal cells present in amniotic fluid and mixtures of body fluids.
- **10**. A kit for simultaneously analyzing short tandem repeat sequences in at least three loci, comprising:
 - a single container containing oligonucleotide primers for each locus in a set of at least three short tandem repeat loci, wherein the at least three short tandem repeat loci in the set comprises at least three loci selected from the group consisting of:

HUMTPOX, HUMTH01 and HUMCD4; HUMTPOX, HUMTH01 and HUMVWFA31;

HUMTPOX, HUMVWFA31 and HUMCSF1PO; HUMHPRTB, HUMFESFPS and HUMVWFA31;

HSAC04 (ACTBP2), HUMCYP19 and HUMPLA2A1;

HUMAPOA2, HUMCYP19 and HUMPLA2A1; HUMCD4, HUMCSF1PO and HUMTH01;

HUMCYP19, HUMFABP and HUMPLA2A1;

HUMCYP19, HUMHPRTB and HUMPLA2A1; HUMHPRTB HUMERSEPS and HUMI IPOL:

HUMHPRTB, HUMFESFPS and HUMLIPOL; HUMF13AO1, HUMFABP and HUMCD4;

HUMHPRTB, HUMBFXIII (F13B) and HUMPLA2A1;

HUMHPRTB, HUMBFXIII (F13B) and HUMTPOX; HUMHPRTB, HUMBFXIII (F13B) and HUMFES-FPS;

HUMBFXIII (F13B), HUMFESFPS and HUMLIPOL; HUMCSF1PO, HUMTPOX and HUMCD4;

HUMBRTB, HUMFESFPS and HUMMYOPK (Myotonic);

HUMCSF1PO, HUMTH01 and HUMCD4;

HUMCSF1PO, HUMTH01 and HUMVWFA31; and HUMHPRTB, HUMBFXIII (F13B) and HUMLIPOL.

11. The kit of claim 10 wherein each of the oligonucleotide primers in the kit is designed to hybridize with an allele of one of the loci in the set of at least two short tandem repeat loci, wherein the sequence of at least one of the primers is selected from the group consisting of:

SEQ ID. NO. 1 and SEQ ID. NO. 2 when one of the loci in the set is HSAC04;

SEQ ID. NO. 3 and SEQ ID. NO. 4 when one of the loci in the set is HUMAPOA2;

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- SEQ ID. NO. 5 and SEQ ID. NO. 6 when one of the loci in the set is HUMCSF1PO;
- SEQ ID. NO. 7 and SEQ ID. NO. 8 when one of the loci in the set is HUMCYP19;
- SEQ ID. NO. 9 and SEQ ID. NO. 10 when one of the loci ⁵ in the set is HUMCD4;
- SEQ ID. NO. 11 and SEQ ID. NO. 12 when one of the loci in the set is HUMF13A01;
- SEQ ID. NO. 13 and SEQ ID. NO. 14 when one of the loci $_{10}$ in the set is HUMBFXIII;
- SEQ ID. NO. 15 and SEQ ID. NO. 16 when one of the loci in the set is HUMFABP;
- SEQ ID. NO. 17 and SEQ ID. NO. 18 when one of the loci in the set is HUMFESFPS;
- SEQ ID. NO. 19 and SEQ ID. NO. 20 when one of the loci in the set is HUMBPRTB;
- SEQ ID. NO. 21 and SEQ ID. NO. 22 when one of the loci in the set is HUMMYOPK (Myotonic);
- SEQ ID. NO. 23 and SEQ ID. NO. 24 when one of the loci in the set is HUMLIPOL;
- SEQ ID. NO. 25 and SEQ ID. NO. 26 when one of the loci in the set is HUMPLA2A1;
- SEQ ID. NO. 27 and SEQ ID. NO. 28 when one of the loci 25 in the set is HUMTH01;
- SEQ ID. NO. 29 and SEQ ID. NO. 30 when one of the loci in the set is HUMTPOX; and
- SEQ ID. NO. 31 and SEQ ID. NO. 32 when one of the loci in the set is HUMVWFA31.
- 12. A method of simultaneously determining the alleles present in a set of short tandem repeat loci from one or more DNA samples, comprising:
 - a) obtaining at least one DNA sample to be analyzed;
 - b) selecting a set of short tandem repeat loci of the DNA sample to be analyzed which can be co-amplified, comprising HUMCSF1PO, HUMTPOX, and HUMTH01:
 - c) co-amplifying the set of short tandem repeat loci in a 40 multiplex amplification reaction, thereby producing a mixture of amplified alleles from each of the co-amplified loci in the set; and
 - d) evaluating the amplified alleles in the mixture to determine the alleles present at each of the co-amplified 45 loci in the set.
- 13. The method of claim 12, wherein the multiplex reaction is carried out using oligonucleotide primer pairs with primer pair sequences comprising: SEQ ID. NO. 5 and SEQ ID. NO. 6; SEQ ID. NO. 29 and SEQ ID. NO. 30; and 50 SEQ ID. NO. 27 and SEQ ID. NO. 28.
- 14. The method of claim 12, wherein the oligonucleotide primer pairs having the sequences SEQ ID. NO. 5 and SEQ ID. NO. 6, and SEQ ID. NO. 29 and SEQ ID. NO. 30 are present in a concentration of about 0.2 μ M, and the oligonucleotide primer pairs SEQ ID. NO. 27 and SEQ ID. NO. 28 are present in a concentration of about 0.6 μ M.
- 15. The method of claim 12, wherein the set of loci co-amplified further comprises HUMVWFA31.
- 16. The method of claim 12, wherein the multiplex 60 reaction is carried out using oligonucleotide primer pairs with primer pair sequences comprising: SEQ ID. NO. 5 and SEQ ID. NO. 6, SEQ ID. NO. 29 and SEQ ID. NO. 30, SEQ ID. NO. 27 and SEQ ID. NO. 28, and SEQ ID. NO. 31 and SEQ ID. NO. 32.
- 17. The method of claim 16, wherein the oligonucleotide primer pairs SEQ ID. NO. 5 and SEQ ID. NO. 6 are present

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- in a concentration of about 1 μ M; oligonucleotide primer pairs SEQ ID. NO. 29 and SEQ ID. NO. 30 are present in a concentration of about 0.15 μ M, oligonucleotide primer pairs SEQ ID. NO. 27 and SEQ ID. NO. 28 are present in a concentration of about 0.2 μ M, and oligonucleotide primer pair SEQ ID. NO. 31 and SEQ ID. NO. 32 are present in a concentration of about 1 μ M.
- 18. The method of claim 12, wherein the amplified alleles are separated by denaturing polyacrylamide gel electrophoresis, and detected by silver staining.
- 19. The method of claim 12, wherein the multiplex amplification reaction includes oligonucleotide primers for each locus in the set of loci selected in step (b), wherein at least one of the oligonucleotide primers for each locus is fluorescently labeled.
- **20**. The method of claim **12**, wherein the set of loci co-amplified further comprises HUMCD4.
- 21. The method of claim 12, wherein the set of loci co-amplified further comprises HUMVWFA31.
- 22. The method of claim 12, wherein the amplified alleles 20 are separated by denaturing polyacrylamide gel electrophoresis, and detected by fluorescent detection.
 - 23. A kit for simultaneously analyzing short tandem repeat sequences in a set of short tandem repeat loci from one or more DNA samples, comprising:
 - a single container containing oligonucleotide primers for each locus in a set of short tandem repeat loci which can be co-amplified, comprising HUMCSF1PO, HUMTPOX, and HUMTH01.
- 24. The kit of claim 23, wherein the kit contains oligonucleotide primers designed to co-amplify the set of short tandem repeat loci, further comprising HUMVWFA31.
 - 25. The kit of claim 23, wherein the kit contains oligonucleotide primers designed to co-amplify the set of short tandem repeat loci, further comprising HUMCD4.
 - 26. The kit of claim 23, wherein each of the oligonucleotide primers in the kit is designed to hybridize with an allele of one of the loci in the set of at least three short tandem repeat loci, wherein at least one of the oligonucleotide primers in the kit has a sequence selected from the group consisting of: SEQ ID. NO. 5, SEQ ID. NO. 6, SEQ ID. NO. 29, SEQ ID. NO. 30, SEQ ID. NO. 27, and SEQ ID. NO. 28.
 - 27. The kit of claim 23, wherein one of each of the pair of oligonucleotide primers in the kit is fluorescently-labeled.
 - 28. A method of simultaneously determining the alleles present in a set of short tandem repeat loci from one or more DNA samples, comprising:
 - a) obtaining at least one DNA sample to be analyzed;
 - b) selecting a set of short tandem repeat loci of the DNA sample to be analyzed which can be co-amplified, comprising HUMTPOX, HUMVWFA31, and HUMCSF1PO;
 - c) co-amplifying the set of short tandem repeat loci in a multiplex amplification reaction, thereby producing a mixture of amplified alleles from each of the co-amplified loci in the set; and
 - d) evaluating the amplified alleles in the mixture to determine the alleles present at each of the co-amplified loci in the set.
 - 29. The method of claim 28, wherein the multiplex reaction is carried out using oligonucleotide primer pairs with at least one primer pair selected from the group of primer pair sequences consisting of: SEQ ID. NO. 29 and SEQ ID. NO. 30; SEQ ID. NO. 31 and SEQ ID. NO. 32; and SEQ ID. NO. 5 and SEQ ID. NO. 6.
 - 30. The method of claim 28, wherein the amplified alleles are separated by denaturing polyacrylamide gel electrophoresis, and detected by silver staining.

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- 31. The method of claim 28, wherein the amplified alleles are separated by denaturing polyacrylamide gel electrophoresis, and detected by fluorescent analysis.
- 32. The method of claim 31, wherein the multiplex amplification reaction includes oligonucleotide primers for 5 each locus in the set of loci selected in step (b), wherein at least one of the oligonucleotide primers for each locus is fluorescently labeled.
- **33**. A kit for simultaneously analyzing short tandem repeat sequences in a set of short tandem repeat loci from 10 one or more DNA samples, comprising:
 - a single container containing oligonucleotide primers for each locus in a set of short tandem repeat loci which can be co-amplified, comprising HUMTPOX, HUMVWFA31, and HUMCSF1PO.
- 34. The kit of claim 33, wherein each of the oligonucleotide primers in the kit is designed to hybridize with an allele of one of the loci in the set of at least three short tandem repeat loci, wherein at least one of the oligonucleotide primers in the kit has a sequence selected from the group ²⁰ consisting of: SEQ ID. NO. 29 and SEQ ID. NO. 30; SEQ ID. NO. 31 and SEQ ID. NO. 32; and SEQ ID. NO. 5 and SEQ ID. NO. 6.
- **35**. A method of simultaneously determining the alleles present in a set of short tandem repeat loci from one or more ²⁵ DNA samples, comprising:
 - a) obtaining at least one DNA sample to be analyzed;
 - b) selecting a set of short tandem repeat loci of the DNA sample to be analyzed which can be co-amplified, comprising HUMBFXIII (F13B), HUMFESFPS, and HUMLIPOL:
 - c) co-amplifying the set of short tandem repeat loci in a multiplex amplification reaction, thereby producing a mixture of amplified alleles from each of the 35 co-amplified loci in the set; and
 - d) evaluating the amplified alleles in the mixture to determine the alleles present at each of the co-amplified loci in the set.

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- 36. The method of claim 35, wherein the multiplex reaction is carried out using oligonucleotide primer pairs with at least one primer pair selected from the group of primer pair sequences consisting of: SEQ ID. NO. 13 and SEQ ID. NO. 14; SEQ ID. NO. 17 and SEQ ID. NO. 18; and SEQ ID. NO. 23 and SEQ ID. NO. 24.
- 37. The method of claim 35, wherein the set of short tandem repeat loci selected for multiplex amplification further comprises HUMHPRTB.
- 38. The method of claim 35, wherein the amplified alleles are separated by denaturing polyacrylamide gel electrophoresis, and detected by silver staining.
- 39. The method of claim 35, wherein the amplified alleles are separated by denaturing polyacrylamide gel electrophoresis, and detected by fluorescent analysis.
- **40**. The method of claim **35**, wherein the multiplex amplification reaction includes oligonucleotide primers for each locus in the set of loci selected in step (b), wherein at least one of the oligonucleotide primers for each locus is fluorescently labeled.
- 41. A kit for simultaneously analyzing short tandem repeat sequences in a set of short tandem repeat loci from one or more DNA samples, comprising:
 - a single container containing oligonucleotide primers for each locus in a set of short tandem repeat loci which can be co-amplified, comprising HUMBFXIII (F13B), HUMFESFPS, and HUMLIPOL.
- 42. The kit of claim 41, wherein each of the oligonucleotide primers in the kit is designed to hybridize with an allele of one of the loci in the set of at least three short tandem 30 repeat loci, wherein at least one of the oligonucleotide primers in the kit has a sequence selected from the group consisting of: SEQ ID. NO. 13 and SEQ ID. NO. 14; SEQ ID. NO. 17 and SEQ ID. NO. 18; and SEQ ID. NO. 23 and SEQ ID. NO. 24.
 - **43**. The kit of claim **41**, wherein the kit contains oligonucleotide primers designed to co-amplify the set of short tandem repeat loci, further comprising HUMHPRTB.

* * * * *

Case: 13-1011 CaseASE-PARITICIDANTINSEONNES Dorangee 12/38/3 Fireaty e07/38/2015 Bed: 07/12/2013

UNITED STATES PATENT AND TRADEMARK OFFICE CERTIFICATE OF CORRECTION

PATENT NO. : 6,221,598 B1

Page 1 of 1

DATED

: April 24. 2001

INVENTOR(S): James W. Schumm et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 12,

Line 20, "DATP" should read -- dATP --.

Column 13,

Line 66, "DATP" should read -- dATP --.

Column 15,

Line 1, "DATP" should read -- dATP --.

Column 38,

Line 53, "HUMBRTB" should read -- HUMHPRTB --.

Column 39,

Line 17, "HUMBPRTB" should read -- HUMHPRTB --.

Signed and Sealed this

Eleventh Day of December, 2001

Attest:

Nicholas P. Ebdici

Attesting Officer

NICHOLAS P. GODICI Acting Director of the United States Patent and Trademark Office Case: 13-1011 Cast ASE-PARITICIPANTISEO IN BY DORANGE 12393 FIREO 12392/20 E3 ed: 07/12/2013

TAB 8

US006479235B1

(12) United States Patent Schumm et al.

(10) Patent No.: US 6,479,235 B1

(45) **Date of Patent:** Nov. 12, 2002

(54) MULTIPLEX AMPLIFICATION OF SHORT TANDEM REPEAT LOCI

(75) Inventors: **James W. Schumm; Cynthia J. Sprecher**, both of Madison, WI (US)

(73) Assignee: Promega Corporation, Madison, WI

(US)

(*) Notice: Subject to any disclaimer, the term of this

patent is extended or adjusted under 35

U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/199,542**

(22) Filed: Nov. 25, 1998

Related U.S. Application Data

(63) Continuation-in-part of application No. 08/632,575, filed on Apr. 15, 1996, now Pat. No. 5,843,660, which is a continuation-in-part of application No. 08/316,544, filed on Sep. 30, 1004

(51) **Int. Cl.**⁷ **1C12Q 1/68**; C12P 19/34; C07H 21/04; C07H 21/02

(52) **U.S. Cl.** **435/6**; 435/91.2; 435/91.5; 536/23.1; 536/24.33

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(List continued on next page.)

Primary Examiner—Stephanie W. Zitomer (74) Attorney, Agent, or Firm—Michael Best & Friedrich LLP; Grady J. Frenchick; Karen B. King

(57) ABSTRACT

Methods and materials are disclosed for use in simultaneously amplifying at least thirteen loci of genomic DNA in a single multiplex reaction, as are methods and materials for use in the analysis of the products of such reactions. Included in the present invention are materials and methods for the simultaneous amplification of at least thirteen short tandem repeat loci, including specific materials and methods for the analysis of thirteen such loci specifically selected by the United States Federal Bureau of Investigation as core loci for use in the Combined DNA Index System (CODIS) database.

24 Claims, 12 Drawing Sheets

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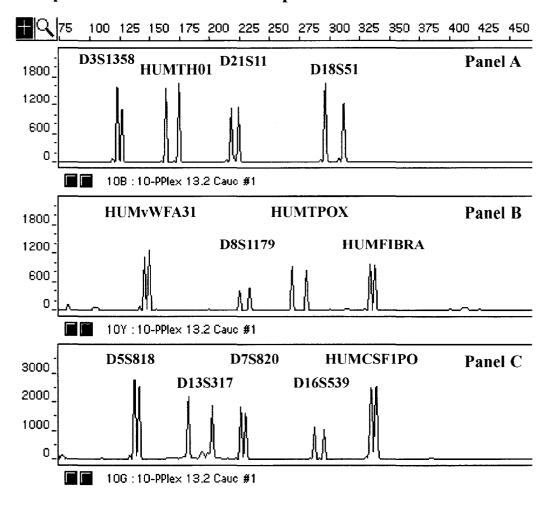
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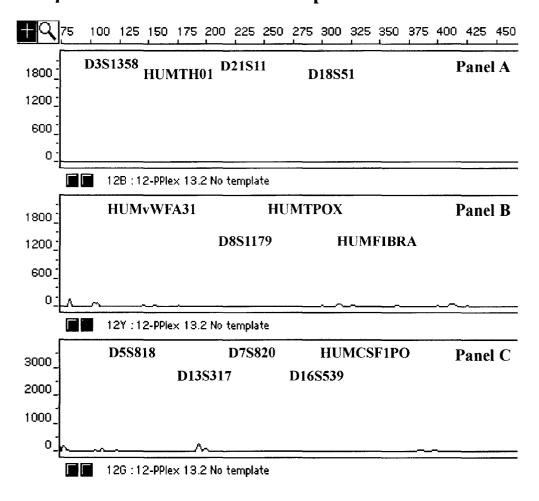
FIG. 1A
Amplification with DNA template



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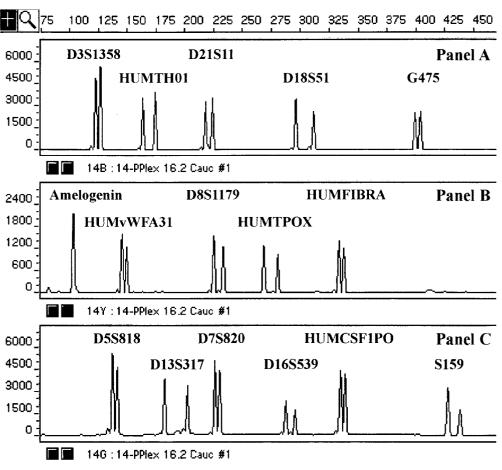
FIG. 1B
Amplification with NO DNA template



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FIG. 2A
Amplification with DNA template

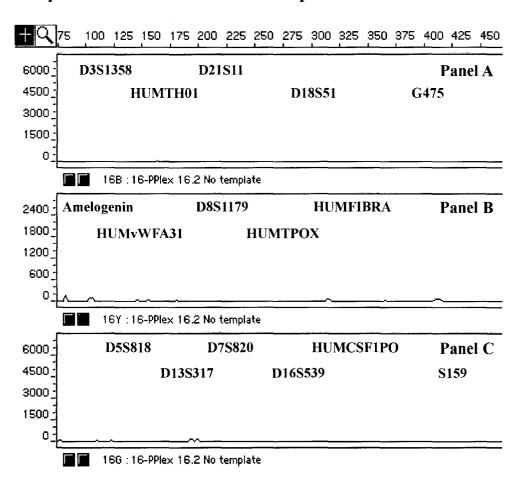


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FIG. 2B

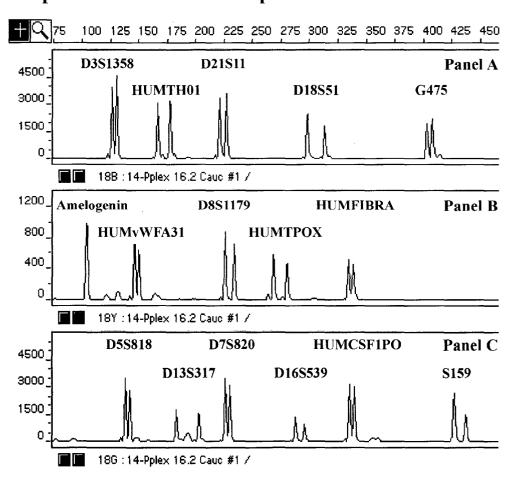
Amplification with NO DNA template



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FIG. 3A
Amplification with DNA template

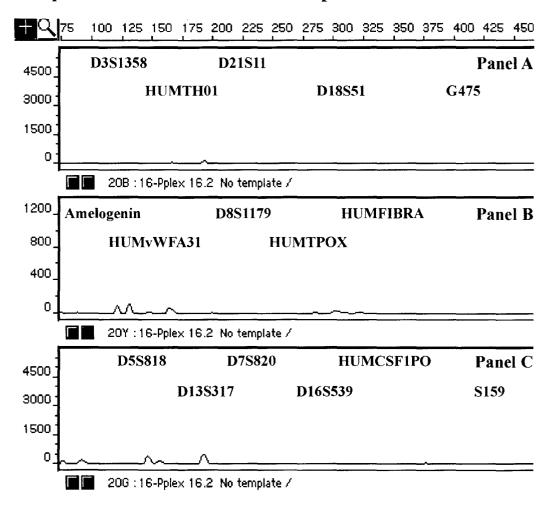


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FIG. 3B

Amplification with NO DNA template



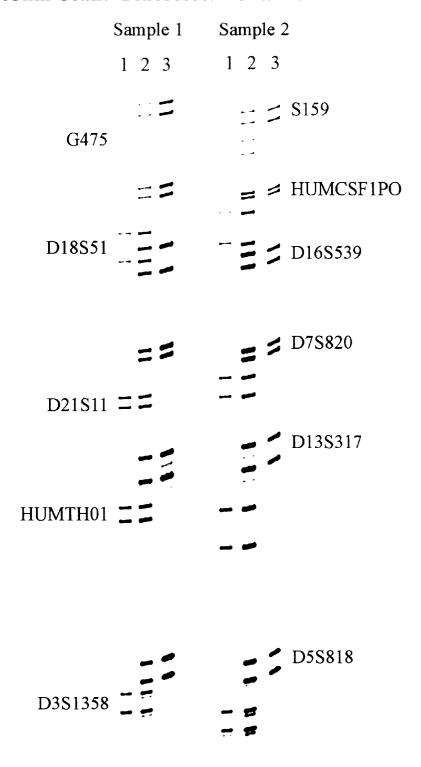
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FIG 4A

505nm Scan: Fluorescein Channel



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FIG 4B

585nm Scan: Tetramethyl Rhodamine Channel

Sample 2 Sample 1 1 2 3 1 2 3

HUMFIBRA --

HUMTPOX --

D8S1179

HUMvWFA31 ...

Amelogenin

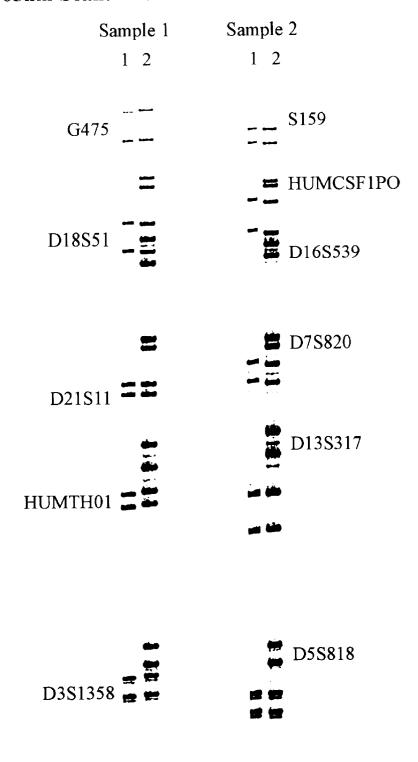
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FIG 5A

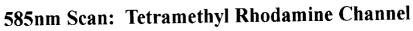
505nm Scan: Fluorescein Channel

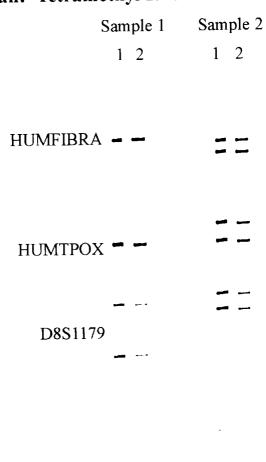


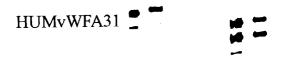
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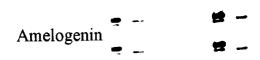
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FIG 5B









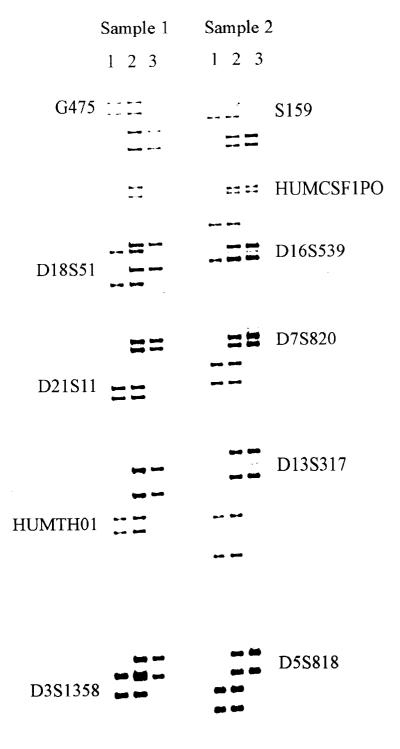
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FIG 6A

505nm Scan: Fluorescein Channel



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FIG 6B

585nm Scan: Tetramethyl Rhodamine Channel

Sample 2 Sample 1

1 2 3 1 2 3

HUMFIBRA --

HUMTPOX --

D8S1179

HUMvWFA31

Amelogenin

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MULTIPLEX AMPLIFICATION OF SHORT TANDEM REPEAT LOCI

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. patent application Ser. No. 08/632,575, filed Apr. 15, 1996, now U.S. Pat. No. 5,843,660, issued Dec. 1, 1998, which is a continuation-in-part of U.S. patent application Ser. No. 08/316,544, filed Sep. 30, 1994. The entire disclosure of ¹⁰ those applications is incorporated by reference herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

FIELD OF THE INVENTION

The present invention is generally directed to the detection of genetic markers in a genomic system. The present invention is more specifically directed to the simultaneous amplification of multiple distinct polymorphic genetic loci using the polymerase chain reaction or other amplification systems to determine, in one reaction, the alleles of each locus contained within the multiplex system.

BACKGROUND OF THE INVENTION

DNA typing is commonly used to identify the parentage of human children, and to confirm the lineage of horses, dogs, other animals, and agricultural crops. DNA typing is also commonly employed to identify the source of blood, saliva, semen, and other tissue found at a crime scenes or other sites requiring identification of human remains. DNA typing is also employed in clinical settings to determine success or failure of bone marrow transplantation and presence of particular cancerous tissues. DNA typing involves the analysis of alleles of genomic DNA with characteristics of interest, commonly referred to as "markers". Most typing methods in use today are specifically designed to detect and analyze differences in the length and/or sequence of one or more regions of DNA markers known to appear in at least two different forms in a population. Such length and/or sequence variation is referred to as "polymorphism." Any region (i.e. "locus") of DNA in which such a variation occurs is referred to as a "polymorphic locus." The methods and materials of the present invention are designed for use in the detection of multiple loci of DNA, some or all of which are polymorphic loci.

Genetic markers which are sufficiently polymorphic with respect to length or sequence have long been sought for use in identity applications, such as paternity testing and identification of tissue samples collected for forensic analysis. The discovery and development of such markers and methods for analyzing such markers have gone through several phases of development over the last several years.

The first identified DNA variant markers were simple base substitutions, i.e. simple sequence polymorphisms, which were most often detected by Southern hybridization assays. For examples of references describing the identification of such markers, designed to be used to analyze restriction endonuclease-digested DNA with radioactive probes, see: Southern, E. M. (1975), J. Mol. Biol. 98(3):503–507; Schumm, et al. (1988), American Journal of Human Genetics 42:143–159; and Wyman, A. and White, R. (1980) Proc. Natl. Acad. Sci, U.S.A. 77:6754–6758.

The next generation of markers were size variants, i.e. length polymorphisms, specifically "variable number of

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tandem repeat" (VNTR) markers (Nakamura Y., et al. (1987), Science 235: 1616–1622; and U.S. Pat. No. 4,963, 663 issued to White et al. (1990); U.S. Pat. No. 5,411,859 continuation of 4,963,663 issued to White et al. (1995)) and "minisatellite" markers (Jeffreys et al. (1985a), Nature 314:67–73; Jeffreys et al. (1985b) Nature 316:76–79., U.S. Pat. No. 5,175,082 for an invention by Jeffreys). Both VNTR and minisatellite markers, contain regions of nearly identical sequences repeated in tandem fashion. The core repeat sequence is 10 to 70 bases in length, with shorter core repeat sequences referred to as "minisatellite" repeats and longer repeats referred to as VNTRs. Different individuals in a human population contain different numbers of the repeats. The VNTR markers are generally more highly polymorphic than base substitution polymorphisms, sometimes displaying up to forty or more alleles at a single genetic locus. However, the tedious process of restriction enzyme digestion and subsequent Southern hybridization analysis are still required to detect and analyze most such markers.

The next advance involved the joining of the polymerase chain reaction (PCR) (U.S. Pat. No. 4,683,202 by Mullis, K. B.) technology with the analysis of VNTR loci (Kasai, K. et al. (1990) Journal Forensic Science 35(5):1196-1200). Amplifiable VNTR loci were discovered, which could be detected without the need for Southern transfer. The amplified products are separated through agarose or polyacrylamide gels and detected by incorporation of radioactivity during the amplification or by post-staining with silver or ethidium bromide. However, PCR can only be used to 30 amplify relatively small DNA segments reliably, i.e. only reliably amplifying DNA segments under 3,000 bases in length Ponce, M & Micol, L. (1992) NAR 20(3):623; Decorte R, et al. (1990) DNA Cell Biol. 9(6):461-469). Consequently, very few amplifiable VNTRs have been developed.

In recent years, the discovery and development of polymorphic short tandem repeats (STRs) as genetic markers has stimulated progress in the development of linkage maps, the identification and characterization of diseased genes, and the simplification and precision of DNA typing. Specifically, with the discovery and development of polymorphic markers containing dinucleotide repeats (Litt and Luty (1989) Am J. Hum Genet 3(4):599-605; Tautz, D (1989) NAR 17:6463-6471; Weber and May (1989) Am J Hum Genet 45 44:388-396; German Pat. No. DE 38 34 636 C2, inventor Tautz, D; U.S. Pat. No. 5,582,979 filed by Weber, L.), STRs with repeat units of three to four nucleotides (Edwards, A., et al. (1991) Am. J. Hum. Genet. 49: 746-756.; Hammond, H. A., et al. (1994) Am. J. Hum. Genet. 55: 175-189; 50 Fregeau, C. J.; and Fourney, R. M. (1993) BioTechniques 15(1): 100-119.; Schumm, J. W. et al. (1994) in The Fourth International Symposium on Human Identification 1993, pp. 177-187 (pub. by Promega Corp., 1994); and U.S. Pat. No. 5,364,759 by Caskey et al.; German Pat. No. DE 38 34 636 C2 by Tautz, D.) and STRs with repeat units of five to seven bases (See, e.g. Edwards et al. (1991) Nucleic Acids Res. 19:4791; Chen et al. (1993) Genomics 15(3): 621-5; Harada et al. (1994) Am. J. Hum. Genet. 55: 175-189; Comings et al. (1995), Genomics 29(2):390-6; and Utah Marker Development Group (1995), Am. J. Genet. 57:619-628; and Jurka and Pethiyagoda (1995) J. Mol. Evol. 40:120-126)), many of the deficiencies of previous methods have been overcome. STR markers are generally shorter than VNTR markers, making them better substrates for amplification 65 than most VNTR markers.

STR loci are similar to amplifiable VNTR loci in that the amplified alleles at each such locus may be differentiated

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based on length variation. Generally speaking STR loci are less polymorphic at each individual locus than VNTR loci. Thus, it is desirable to amplify and detect multiple STR systems in a single amplification reaction and separation to provide information for several loci simultaneously. Systems containing several loci are called multiplex systems and many such systems containing up to 11 separate STR loci have been described. See, e.g., Proceedings: American Academy of Forensic Sciences (Feb. 9–14, 1998), Schumm, James W. et al., p. 53, B88; Id., Gibson, Sandra D. et al., p. 10 53, B89; Id., Lazaruk, Katherine et al., p. 51, B83; Sparkes, R. et al., Int J Legal Med (1996) 109:186-194; AmpFlSTR Profiler™ PCR Amplification Kit User's Manual (1997), pub by Perkin-Elmer Corp, i-viii and 1-1 to 1-10; AmpFISTR Profiler Plus™ PCR Amplification Kit User's Manual (1997), pub by Perkin-Elmer Corp., i viii and 1-1 to 1-10; AmpFlSTR COfiler™ PCR Amplification Kit User Bulletin (1998), pub by Perkin-Elmer Corp. i-iii and 1-1 to 1-10; 9th International Symposium on Human Identification (Oct. 7-10, 1998), pub. by Promega Corp., Staub, Rick W. et al., 20 Poster Abstract 15; Id., Willard, Jeanne M. et al., Poster Abstract 73; and Id., Walsh, P. Sean, et al., Speaker Abstract for 8:50am-9:20am, Thursday, Oct. 8, 1998.

Amplification protocols with STR loci can be designed to produce small products, generally from 60 to 500 base pairs 25 (bp) in length, and alleles from each locus are often contained within a range of less than 100 bp. This allows simultaneous electrophoretic analysis of several systems on the same gel or capillary electrophoresis by careful design of PCR primers such that all potential amplification products 30 from an individual system do not overlap the range of alleles of other systems. Design of these systems is limited, in part, by the difficulty in separating multiple loci in a single gel or capillary. This occurs because there is spacial compression of fragments of different sizes, especially longer fragments 35 in gels or capillaries, i.e., commonly used means for separation of DNA fragments by those skilled in the art.

The United States Federal Bureau of Investigation ("FBI") has established and maintains a Combined DNA Index System ("CODIS"), a database of DNA typing infor- 40 invention which is directed to a method and materials for mation. Local, state, and national law enforcement agencies use the CODIS system to match forensic DNA evidence collected at crime scenes with DNA information in the database. CODIS and other national database systems have proven to be an effective tool for such agencies to use in 45 solving violent crimes. (See, e.g. Niezgoda, Stephen, in Cambridge Healthtech Institute's Second Annual Conference on DNA Forensics: Science, Evidence, and Future Prospects (Nov. 17-18, 1998), pp. 1-21.; Niezgoda, Stephen in Proceedings From The Eighth International Symposium 50 on Human Identification 1997, pub. by Promega Corporation (1998), pp 48-49; Frazier, Rachel R. E. et al. Id., pp. 56-60; Niezgoda, S. J. Profiles in DNA 1(3): 12-13; Werrett, D. J. and Sparkes, R. in Speaker Abstracts: 9th International Symposium on Human Identification (Oct. 7–10, 1998) pp. 55 5-6). Until recently, only restriction fragment length polymorphism ("RFLP") data obtained from the analysis of particular VNTR loci was considered a core component in the database. The FBI has recently identified thirteen polymorphic STR loci for inclusion in the CODIS database. The 60 thirteen CODIS STR loci are HUMCSF1PO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMFIBRA, HUMTH01, HUMTPOX, and HUMvWFA31. (Budowle, Bruce and Moretti, Tamyra in Speaker Abstracts: 9th International Symposium on Human 65 Identification (Oct. 7-10, 1998) pp. 7-8). Both VNTR and STR marker data are currently maintained in the CODIS

database. (See, e.g. Niezgoda, Stephen in Second Annual Conference on DNA Forensics, supra). Until the present invention, the number of loci which could be co-amplified in a single reaction, and analyzed thereafter was limited. Specifically, no materials or methods had been developed for use in multiplex amplification of thirteen or more STR loci, much less the thirteen polymorphic STR loci identified for use in the CODIS database.

The materials and methods of the present method are designed for use in multiplex analysis of particular polymorphic loci of DNA of various types, including singlestranded and double-stranded DNA from a variety of different sources. The present invention represents a significant improvement over existing technology, bringing increased power of discrimination, precision, and throughput to DNA profiling for linkage analysis, criminal justice, paternity testing, and other forensic, medical, and genetic identification applications.

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide a method and materials for the simultaneous amplification of sets of loci, which include multiple distinct polymorphic short tandem repeat (STR) loci, in a single multiplex reaction, using PCR or other amplification systems in combination with gel electrophoresis, capillary electrophoresis or other separation and detection methods to analyze and compare the relative lengths of the alleles of each locus amplified in the multiplex reaction. Multiplex analysis of the sets of loci disclosed herein has not been previously described in the prior art. There has also not been any previous description of the sequences for many of the primers disclosed herein below, all of which are shown to be useful in multiplex amplification of the sets of loci disclosed.

It is also an object of the present invention to provide a method, a kit, and primers specific for multiplex amplifications comprising specified loci.

These and other objects are addressed by the present simultaneously analyzing or determining the alleles present at each individual locus of each multiplex. In general, the method of this invention comprises the steps of (a) obtaining at least one DNA sample to be analyzed, wherein the DNA sample has at least thirteen loci which can be co-amplified; (b) co-amplifying the at least thirteen loci of the DNA sample; and (c) detecting the amplified materials in a fashion which reveals the polymorphic nature of the systems employed.

In one embodiment, the present invention is a method of simultaneously determining the alleles present in a set of loci from one or more DNA samples, comprising the steps

- (a) obtaining at least one DNA sample to be analyzed;
- (b) selecting a set of loci of the DNA sample, comprising at least thirteen short tandem repeat loci which can be co-amplified;
- (c) co-amplifying the loci in the set in a multiplex amplification reaction, wherein the product of the reaction is a mixture of amplified alleles from each of the co-amplified loci in the set; and
- (d) evaluating the amplified alleles in the mixture to determine the alleles present at each of the loci analyzed in the set within the DNA sample.

At least four of the at least thirteen short tandem repeat loci are preferably selected from the group of loci consisting of:

D3S1539, D4S2368, D5S818, D7S820, D9S930, D10S1239, D13S317, D14S118, D14S548, D14S562, D16S490, D16S539, D16S753, D17S1298, D17S1299, D19S253, D20S481, D22S683, HUMCSF1PO, HUMTPOX, HUMTH01, HUMF13A01, 5 HUMBFXIII, HUMLIPOL, HUMvWFA31.

In another embodiment of the invention, the set of loci selected in step (b) of In another embodiment of the invention, the set of loci selected in step (b) of the method comprises thirteen CODIS STR loci (i.e., D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, and HUMvWFA31) which can be co-amplified and analyzed by themselves, or with additional loci using methods of the present invention.

In a further aspect, this invention is a kit for simultaneously analyzing a set of loci of genomic DNA, comprising oligonucleotide primers for co-amplifying a set of loci of the genomic DNA to be analyzed, wherein the set of loci comprises at least thirteen short tandem repeat loci which 20 can be co-amplified in the same multiplex reaction, and wherein the primers are in one or more containers. More preferably, the kit comprises oligonucleotide primer pairs for co-amplifying a set of at least thirteen loci of human genomic DNA, the set of loci comprising D3S1358, 25 D5S818, D7S820, D8S1179, D 13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, and HUMvWFA31.

In yet a further aspect, the invention is primer sequences and primer pairs for amplifying specific loci of human DNA. 30 Use of the primers and primer pairs of this invention for multiplex analysis of human DNA is demonstrated herein, below. The primers of this invention are suitable for use in the method of this invention, wherein they can be used in labeled form, as noted below, to assist the evaluation step of 35 the method.

The approaches specified in the present invention produce savings of time, labor, and materials in the analysis of loci contained within the multiplexes. The method of the present invention allows thirteen or more, even as many as sixteen 40 or more, loci to be co-amplified in one tube using a single amplification reaction, instead of amplifying each locus independently in separate tubes or in smaller groups of loci.

The present invention has specific use in the field of forensic analysis, paternity determination, monitoring of 45 bone marrow transplantation, linkage mapping, and detection of genetic diseases and cancers. By allowing thirteen methods of the present invention significantly increase the certainty with which one can match DNA prepared from different samples from the same individual. The need to 50 match or distinguish accurately between samples containing very small amounts of DNA is particularly acute in forensics applications, where many convictions (and acquittals) turn on DNA typing analysis.

Scientists, particularly forensic scientists, have long 55 appreciated the need to analyze multiple polymorphic loci of DNA in order to ensure that a match between two samples of DNA is statistically significant. (Presley, L. A. et al., in *The Third International Symposium on Human Identification* 1992, pp. 245–269 (pub. by Promega Corp., 1993); Bever, 60 R. A., et al., in *The Second International Symposium on Human Identification* 1991, pp.103–128. (pub. by Promega Corp., 1992)). However, until this invention, one could not simultaneously analyze thirteen or more STR loci in a single reaction. To realize the importance of such multiplexing 65 capabilities, it helps to understand some of the mathematics behind DNA typing analysis.

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For purposes of illustration, suppose every STR locus has a genotype (i.e., pattern of two alleles) frequency of one in ten. In other words, suppose that the chance of two randomly selected individuals have a matching type for a single STR is 1/10. However, if two different STR loci are analyzed, the chance of a random match with both systems is 1/100. If three STR loci are analyzed, the chances of a random match with each of the three systems is 1/1,000 and so on. Consequently, it is easy to see how increasing the number of STR loci analyzed reduces the likelihood of random matches within the general population, thereby increasing the chance one can accurately identify a suspect's presence at a crime scene by comparing the individual's type with crime scene evidence. Similar reasoning can be used to conclude that the 15 method of this invention also would increase the likelihood of accurately identifying a suspected father in a paternity case, of correctly matching bone marrow tissue, of developing significant results from linkage mapping studies, and of detecting genetic diseases and cancers.

Further objects, features, and advantages of the invention will be apparent from the following best mode for carrying out the invention and the illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a plot of the output of three-color fluorescent detection of the products of simultaneous amplification of the loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, and HUMvWFA31 of a sample of human genomic DNA, as detected with the ABI PRISM® 310 Genetic Analyzer in Example 1.

FIG. 1B is a plot of the output of three-color fluorescent detection of a control sample processed the same way as FIG. 1A, with no genomic DNA in the amplification reaction

FIG. 2A is a plot of the output of three-color fluorescent detection of the products of simultaneous amplification of the loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21 S11, HUMCSF1PO HUMFIBRA, HUMTH01, HUMTPOX, HUMvWFA31, G475, S159, and Amelogenin of a sample of human genomic DNA, as detected with the ABI PRISM® 310 Genetic Analyzer in Example 2.

FIG. 2B is a plot of the output of three-color fluorescent detection of a control sample processed the same way as FIG. 2A, with no genomic DNA substrate in the amplification reaction.

FIG. 3A is a plot of the output of three-color fluorescent detection of the products of simultaneous amplification of the loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, HUMvWFA31, G475, S159, and Amelogenin of a sample of human genomic DNA, as detected with an ABI PRISM® 377 DNA Sequencer in Example 3.

FIG. 3B is a plot of the output of three-color fluorescent detection of a control sample processed the same way as FIG. 3A, with no genomic DNA substrate in the amplification reaction.

FIGS. 4A and 4B are laser printed images of the results of fluorescent detection of the products of simultaneous amplification of the loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, HUMvWFA31, G475, S159, and Amelogenin as detected using the fluorescein channel (FIG. 4A) and carboxy-tetramethylrhodamine

channel (FIG. 4B) of a Hitachi FMBIO® II Fluorescent Scanner, as described in Example 4.

FIGS. 5A and 5B are laser printed images of the results of fluorescent detection of the products of simultaneous amplification of the loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTHO1, HUMTPOX, HUMvWFA31, G475, S159, and Amelogenin as detected using the fluorescein channel (FIG. 5A) and carboxy-tetramethylrhodamine channel (FIG. 5B) of a Hitachi FMBIO® II Fluorescent Scanner, as described in Example 5.

FIGS. 6A and 6B are laser printed images of the results of fluorescent detection of the products of simultaneous amplification of the loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, HUMvWFA31, C221, S159, and Amelogenin as detected using the fluorescein channel (FIG. 6A) and carboxy-tetramethylrhodamine channel (FIG. 6B) of a Hitachi FMBIO® II Fluorescent Scanner, as described in Example 6.

DETAILED DESCRIPTION OF THE INVENTION

A. Definitions

The following definitions are intended to assist in providing a clear and consistent understanding of the scope and detail of the following terms, as used to describe and define the present invention:

"Allelic ladder": a standard size marker consisting of amplified alleles from the locus.

"Allele": a genetic variation associated with a segment of 30 DNA, i.e., one of two or more alternate forms of a DNA sequence occupying the same locus.

"Biochemical nomenclature": standard biochemical nomenclature is used herein in which the nucleotide bases are designated as adenine (A); thymine (T); guanine (G); and Cytosine (C). Corresponding nucleotides are, for example, deoxyguanosine-5'-triphosphate (dGTP).

"DNA polymorphism": the condition in which two or more different nucleotide sequences in a DNA sequence coexist in the same interbreeding population.

"Locus" or "genetic locus": a specific position on a chromosome. Alleles of a locus are located at identical sites on homologous chromosomes.

"Locus-specific primer": a primer that specifically hybridizes with a portion of the stated locus or its complementary strand, at least for one allele of the locus, and does not hybridize efficiently with other DNA sequences under the conditions used in the amplification method.

"Pentanucleotide tandem repeat": a subclass of the STR polymorphisms defined below. Unless specified otherwise, 50 the term "pentanucleotide tandem repeat" encompasses perfect STRs wherein the repeat unit is a five base sequence, and imperfect STRs wherein at least one repeat unit is a five base repeat.

"Polymerase chain reaction" or "PCR": a technique in 55 which cycles of denaturation, annealing with primer, and extension with DNA polymerase are used to amplify the number of copies of a target DNA sequence by approximately 10⁶ times or more. The polymerase chain reaction process for amplifying nucleic acid is covered by U.S. Pat. 60 Nos. 4,683,195 and 4,683,202, which are incorporated herein by reference for a description of the process.

"Polymorphic short tandem repeat loci": STR loci, defined below, in which the number of repetitive sequence elements (and net length of sequence) in a particular region 65 of genomic DNA varies from allele to allele, and from individual to individual.

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"Polymorphism information content" or "PIC": a measure of the amount of polymorphism present at a locus (Botstein et al., 1980). PIC values range from 0 to 1.0, with higher values indicating greater degrees of polymorphism. This measure generally displays smaller values than the other commonly used measure, i.e., heterozygosity. For markers that are highly informative (heterozygosities exceeding about 70%), the difference between heterozygosity and PIC is slight.

"Primer": a single-stranded oligonucleotide or DNA fragment which hybridizes with a DNA strand of a locus in such a manner that the 3' terminus of the primer may act as a site of polymerization using a DNA polymerase enzyme.

"Primer pair": two primers including, primer 1 that hybridizes to a single strand at one end of the DNA sequence to be amplified and primer 2 that hybridizes with the other end on the complementary strand of the DNA sequence to be amplified.

"Primer site": the area of the target DNA to which a 20 primer hybridizes.

"Short tandem repeat loci" or "STR loci": regions of genomic DNA which contain short, repetitive sequence elements of 3 to 7 base pairs in length. The term STR also encompasses a region of genomic DNA wherein more than a single three to seven base sequence is repeated in tandem or with intervening bases, provided that at least one of the sequences is repeated at least two times in tandem. Each sequence repeated at least once within an STR is referred to herein as a "repeat unit."

The sequences of the STR loci analyzed using the materials and methods of the present invention can be divided into two general categories, perfect and imperfect. The term "perfect" STR, as used herein, refers to a region of doublestranded DNA containing a single three to seven base repeat unit repeated in tandem at least two times, e.g. (AAAAT)₂. The term "imperfect" STR, as used herein, refers to a region of DNA containing at least two tandem repeats of a perfect repeat unit and at least one repeat of an imperfect repeat unit, wherein the imperfect repeat unit consists of a DNA 40 sequence which could result from one, two, three, or four base insertions, deletions, or substitutions in the sequence of the perfect repeat unit, e.g. (AAAAT)₁₂(AAAAAT)₅AAT (AAATT)₄. Every imperfect STR sequence contains at least one perfect STR sequence. Specifically, every STR sequence, whether perfect or imperfect, includes at least one repeat unit sequence appearing at least two times in tandem, a repeat unit sequence which can be represented by formula

$$(A_w G_x T_v C_z)_n \tag{I}$$

wherein A, G, T, and C represent the nucleotides which can be in any order; w, x, y and z represent the number of each nucleotide in the sequence and range from 0 to 7 with the sum of w+x+y+z ranging between 3 and 7; and n represents the number of times the sequence is tandemly repeated and is at least 2.

B. Selection of Multiplex Reaction Components

The method of the present invention contemplates selecting an appropriate set of loci, primers, and amplification protocols to generate amplified alleles from multiple co-amplified loci which preferably do not overlap in size or, more preferably, which are labeled in a way which enables one to differentiate between the alleles from different loci which overlap in size. In addition, this method contemplates the selection of short tandem repeat loci which are compatible for use with a single amplification protocol. The specific combinations of loci described herein are unique in this

application. Combinations of loci may be rejected for either of the above two reasons, or because, in combination, one or more of the loci do not produce adequate product yield, or fragments which do not represent authentic alleles are produced in this reaction.

Successful combinations in addition to those disclosed herein can be generated by trial and error of locus combinations, by selection of primer pair sequences, and by adjustment of primer concentrations to identify an equilibrium in which all included loci may be amplified. Once the method and materials of this invention are disclosed, various methods of selecting loci, primer pairs, and amplification techniques for use in the method and kit of this invention are likely to be suggested to one skilled in the art. All such methods are intended to be within the scope of the appended claims.

Of particular importance in the practice of the method of this invention is the size range of amplified alleles produced from the individual loci which are co-amplified in the multiplex amplification reaction step. For ease of analysis with current technologies, systems which can be detected by amplification of fragments smaller than 500 bases are most preferable.

Practice of the method of the present invention begins with selection of a set of loci comprising at least thirteen STR loci, which can be co-amplified in a single multiplex ²⁵ amplification reaction. Selection of loci and oligonucleotide primers used to amplify the loci in the multiplex amplification reaction of the present method is described herein below, and illustrated in the Examples below.

C. Use of Multiplexes of Three Loci to Develop Multiplexes 30 Using More than Three Loci

Any one of a number of different techniques can be used to select a set of loci for use in the present invention. One preferred technique for developing useful sets of loci for use in this method of analysis is described below. Once a multiplex containing three STR loci is developed, it may be used as a core to create multiplexes containing more than three loci. New combinations of more than three loci can, thus, be created which include the first three loci. For example, the core multiplex containing loci D7S820, D13S317, and D5S818 was used to generate derivative multiplexes of:

D16S539, D7S820, D13S317, and D5S818;

HUMCSF1PO, HUMTPOX, D16S539, D7S820, D13S317, and D5S818;

HUMCSF1PO, HUMTPOX, HUMTH01, D16S539, D7S820, D13S317, and D5S818;

HUMCSF1PO, HUMTPOX, HUMTH01, HUMvWFA31, D16S539, D7S820, D13S317, and D5S818;

D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, and HUM-vWA31:

S159, HUMCSF1PO, D16S539, D7S820, D13S317, and D5S818:

D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, and HUM-60 vWFA31; and

D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, HUMvWFA31, G475, S159, and Amelogenin.

It is contemplated that core sets of loci can be used to generate other appropriate derivative sets of STR loci for 10

multiplex analysis using the method of this invention. Regardless of what method is used to select the loci analyzed using the method of the present invention, all the loci selected for multiplex analysis share the following characteristics: (1) they produce sufficient amplification product to allow evaluation; (2) they generate few if any artifacts due to the addition (or lack of addition) of a base to the amplified alleles during the multiplex amplification step; (3) they generate few, if any, artifacts due to premature termination of amplification reactions by a polymerase; and (4) they produce little or no "trailing" bands of smaller molecular weight from consecutive single base deletions below a given authentic amplified allele. See, e.g., Schumm et al., Fourth International Symposium on Human Identification 1993, pp. 15 177–187 (pub. by Promega Corp., 1994).

The same technique used to identify the set of at least three loci, described above, can be applied to select thirteen or more loci of human genomic DNA or multiplex analysis, according to a preferred embodiment of the method of analysis of the present invention. Any set of loci identified as described above is suitable for multiplex analysis in accordance with the present invention, provided the set of loci comprises at least thirteen STR loci. More preferably, at least four of the at least thirteen STR loci analyzed according to the present invention are selected from the group of loci consisting of:

D3S1539, D4S2368, D5S818, D7S820, D9S930, D10S1239, D13S317, D14S118, D14S548, D14S562, D16S490, D16S539, D16S753, D17S1298, D17S1299, D19S253, D20S481, D22S683, HUMCSF1PO, HUMTPOX, HUMTH01, HUMF13A01, HUMBFXIII, HUMLIPOL, and HUMvWFA31

Even more preferably, the set of loci analyzed according to the present invention includes all thirteen CODIS loci, i.e. D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D2IS11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, and HUMvWFA31.

At least one of the loci selected for co-amplification in the present multiplex reaction is preferably an STR locus with 40 a repeat unit of five to seven bases or base pairs in length, more preferably an STR locus with a pentanucleotide repeat. As is demonstrated in U.S. patent application Ser. No. 09/018,584, which is incorporated by reference herein, loci with such intermediate length repeats can be amplified with 45 minimal incidence of artifacts, e.g. due to repeat slippage. Three such loci with pentanucleotide repeats, G475, C221 and S159, are included in the sets of loci identified immediately above. The terms "G475", "C221", and "S159", as used herein, refer to names assigned to pentanucleotide 50 repeat loci identified, as described in U.S. patent application Ser. No. 09/018,584, incorporated by reference above. Each name corresponds to a clone from which each pentanucleotide locus was identified. The sequence of the G475 clone, described therein as SEQ ID NO:34, is identified herein as SEQ ID NO:108. The sequence of the C221 clone, described therein as SEQ ID NO:2, is identified herein as SEQ ID NO:109. The sequence of the S159 clone, described therein as SEQ ID NO: 26, is identified herein as SEQ ID NO:110. Individual primers and primer pairs identified for use in amplifying G475, C221, and S159 therein can also be used to amplify the same loci in the sets of at least thirteen loci co-amplified and analyzed according to the present invention.

The set of loci selected for co-amplification and analysis according to the invention preferably further comprises at least one locus in addition to the at least thirteen STR loci. The additional locus preferably includes a sequence

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polymorphism, or another feature which identifies a particular characteristic which separates the DNA of an individual from the DNA of other individuals in the population. The additional locus more preferably is a locus which identifies the gender of the source of the DNA sample analyzed. When 5 the DNA sample is human genomic DNA, a gender identifying locus such as the Amelogenin locus is preferably selected for co-amplification and analysis according to the present method. The Amelogenin locus is identified by GenBank as HUMAMELY (when used to identify a locus on 10 the Y chromosome contained in male DNA) or as HUMA-MELX (when used to identify a locus on the X chromosome in male or female DNA). When the Amelogenin locus is co-amplified in the same multiplex amplification reaction as the set of at least thirteen short tandem repeat loci, the 15 all alleles and loci is generally not achieved. sequence of at least one of the primers used to amplify this particular locus in the multiplex amplification reaction preferably has a sequence selected from: SEQ ID NO:86, SEQ ID NO:105, and SEQ ID NO:87.

D. Selection of Primers

Once a set of loci for co-amplification in a single multiplex reaction is identified, one can determine primers suitable for co-amplifying each locus in the set. Care should be used in selecting the sequence of primers used in the multiplex reaction. Inappropriate selection of primers can 25 produce several undesirable effects such as lack of amplification, amplification at multiple sites, primer dimer formation, undesirable interaction of primer sequences from different loci, production of alleles from one locus which overlap with alleles from another, or the need for amplifi- 30 cation conditions or protocols for the different loci which are incompatible in a multiplex. Primers used in the present method or included in the present kits of the invention are preferably selected according to the following selection process.

Primers are preferably developed and selected for use in the multiplex systems of the invention by employing a re-iterative process of selecting primer sequences, mixing the primers for co-amplification of the selected loci, co-amplifying the loci, then separating and detecting the 40 amplified products. Initially, this process often produces the amplified alleles in an imbalanced fashion (i.e., higher product yield for some loci than for others) and may also generate amplification products which do not represent the alleles themselves. These extra fragments may result from 45 any number of causes described above.

To eliminate such extra fragments from the multiplex systems, individual primers from the total set are used with primers from the same or other loci to identify which primers contribute to the amplification of the extra frag- 50 ments. Once two primers which generate one or more of the fragments are identified, one or both contributors are modified and retested, either in a pair alone or in the multiplex system (or a subset of the multiplex system). This process is repeated until evaluation of the products yields amplified 55 alleles with no or an acceptable level of extra fragments in the multiplex system.

On occasion, extra fragments can be eliminated by labeling the opposite primer in a primer pair. This change reveals the products of the opposing primer in the detection step. 60 This newly labeled primer may amplify the true alleles with greater fidelity than the previously labeled primer generating the true alleles as a greater proportion of the total amplification product.

The determination of primer concentration may be per- 65 formed either before or after selection of the final primer sequences, but is preferably performed after that selection.

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Generally, increasing primer concentration for any particular locus increases the amount of product generated for that locus. However, this is also a re-iterative process because increasing yield for one locus may decrease it for one or more other loci. Furthermore, primers may interact directly affecting yield of the other loci. Linear increases in primer concentration do not necessarily produce linear increases in product yield for the corresponding locus.

Locus to locus balance is also affected by a number of parameters of the amplification protocol such as the amount of template used, the number of cycles of amplification, the annealing temperature of the thermal cycling protocol, and the inclusion or exclusion of an extra extension step at the end of the cycling process. Absolutely even balance across

The process of multiplex system development may also be a re-iterative process in another sense described, above. That is, it is possible, first, to develop a multiplex system for a small number of loci, this system being free or nearly free of extra fragments from amplification. Primers of this system may be combined with primers for one or more additional loci. This expanded primer combination may or may not produce extra fragments from amplification. In turn, new primers may be introduced and evaluated.

One or more of the re-iterative selection processes described above are repeated until a complete set of primers is identified which can be used to co-amplify the at least thirteen loci selected for co-amplification as described above. It is understood that many different sets of primers may be developed to amplify a particular set of loci.

Synthesis of the primers used in the present method can be conducted using any standard procedure for oligonucleotide synthesis known to those skilled in the art. At least one primer for each locus is preferably covalently attached to a dye label, as described in Section F, below.

Table 1, below, provides a list sequences of primers which have been determined to be suitable for use in amplifying the corresponding polymorphic STR loci listed therein. At least one primer listed in Table 1 is preferably used to amplify at least one of the loci selected for co-amplification and analysis as described above. It is understood that other primers could be identified which are suitable for simultaneous amplification of the loci listed below.

TABLE 1

Locus	Primer SEQ ID NO:'s
D7S820	1, 2, 80 and 81
D13S317	3, 4, 82 and 83
D5S818	5, 6, 84 and 85
D3S1539	7, 8 and 49
D17S1298	9 and 10
D20S481	11, 12, 52 and 53
D9S930	13, 14, 55 and 61
D10S1239	15, 16 and 54
D14S118	17 and 18
D14S562	19 and 20
D14S548	21 and 22
D16S490	23 and 24
D16S753	25 and 26
D17S1299	27 and 28
D16S539	29, 30, 58, 79 and 97
D22S683	31 and 32
HUMCSF1PO	33, 34, 77, 78 and 98
HUMTPOX	35, 36, 72 and 73
HUMTH01	37, 38, 66, 67 and 103
HUMvWFA31	39, 40, 59, 60 and 76
HUMF13A01	41 and 42
HUMFESFPS	43 and 44
HUMBFXIII	45 and 46

TABLE 1-continued

Locus	Primer SEQ ID NO:'s
HUMLIPOL	47 and 48
D19S253	50 and 51
D4S2368	56 and 57
D18S51	62, 63, 101 and 102
D21S11	64 and 65
D351358	68, 69 and 106
HUMFIBRA	70, 71 and 107
D8S1179	74, 75 and 104
G475	88, 89 and 94
S159	90, 91, 92, 93, 95 and 96
C221	99 and 100

E. Preparation of DNA Samples

Samples of genomic DNA can be prepared for use in the method of this invention using any method of DNA preparation which is compatible with the amplification of DNA. Many such methods are known by those skilled in the art. Examples include, but are not limited to DNA purification by phenol extraction (Sambrook, J., et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp. 9.14-9.19), and partial purification by salt precipitation (Miller, S. et al. (1988) Nucl. Acids Res. 16:1215) or chelex 25 (Walsh et al., (1991) BioTechniques 10:506-513, Comey, et al., (1994) Forensic Sci. 39:1254) and the release of unpurified material using untreated blood (Burckhardt, J. (1994) PCR Methods and Applications 3:239-243, McCabe, 1:99-106, Nordvag, Bjørn-Yngvar (1992) BioTechniques 12:4 pp. 490-492).

When the at least one DNA sample to be analyzed using the method of this invention is human genomic DNA, the DNA is preferably prepared from tissue, selected from the 35 group consisting of blood, semen, vaginal cells, hair, saliva, urine, bone, buccal samples, amniotic fluid containing placental cells or fetal cells, chorionic villus, and mixtures of any of the tissues listed above.

use in the method of the present invention, using any standard method of DNA quantification known to those skilled in the art. In such cases, the DNA concentration is preferably determined by spectrophotometric measurement as described by Sambrook, J., et al. (1989), supra, Appendix 45 E.5, or fluorometrically using a measurement technique such as that described by Brunk C. F., et al. (1979), Anal Biochem 92: 497-500. The DNA concentration is more preferably measured by comparison of the amount of hybridization of DNA standards with a human-specific probe such as that 50 described by Waye, J. S., et al. (1991) "Sensitive and specific quantification of human genomic deoxyribonucleic acid (DNA) in forensic science specimens: casework examples," J. Forensic Sci., 36:1198-1203. Use of too much template DNA in the amplification reactions can produce artifacts 55 which appear as extra bands which do not represent true

F. Amplification of DNA

Once a sample of genomic DNA is prepared, the targeted loci can be co-amplified in the multiplex amplification step of the present method. Any one of a number of different amplification methods can be used to amplify the loci, including, but not limited to, polymerase chain reaction (PCR) (Saiki, R. K., et al. (1985), Science 230: 1350-1354), transcription based amplification (Kwoh, D. Y., and Kwoh, 65 T. J. (1990), American Biotechnology Laboratory, October, 1990) and strand displacement amplification (SDA)

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(Walker, G. T., et al. (1992) Proc. Natl. Acad. Sci., U.S.A. 89: 392–396). Preferably, the DNA sample is subjected to PCR amplification using primer pairs specific to each locus in the set. Reference is made to the Sequence Listing at the end of 5 this specification for details of the primer sequences used in the Examples below, some of which sequences are alternative embodiments of this invention.

At least one primer for each locus is preferably covalently attached to a dye label, more preferably a fluorescent dye 10 label. The primers and dyes attached thereto are preferably selected for the multiplex amplification reaction, such that alleles amplified using primers for each locus labeled with one color do not overlap the alleles of the other loci in the set co-amplified therein using primers labeled with the same 15 color, when the alleles are separated, preferably, by gel or capillary electrophoresis.

In a particularly preferred embodiment of the method of the present invention, at least one primer for each locus co-amplified in the multiplex reaction is labeled with a fluorescent label prior to use in the reaction. Fluorescent labels suitable for attachment to primers for use in the present invention are commercially available. See, e.g. fluorescein and carboxy-tetramethylrhodamine labels and their chemical derivatives from PE Biosystems and Molecular Probes. Most preferably, at least three different labels are used to label the different primers used in the multiplex amplification reaction. When a size marker is included to evaluate the multiplex reaction, the primers used to prepare the size marker are preferably labeled with a different label Edward R. B., (1991) PCR Methods and Applications 30 from the primers used to amplify the loci of interest in the reaction.

Details of the most preferred amplification protocol for each of the most preferred combinations of loci for use in the method of this invention are given in the Examples below. Reference is also made to the Examples for additional details of the specific procedure relating to each multiplex. The sequences of the locus-specific primers used in the Examples include a number of nucleotides which, under the conditions used in the hybridization, are sufficient to hybrid-Optionally, DNA concentrations can be measured prior to 40 ize with an allele of the locus to be amplified and to be essentially free from amplification of alleles of other loci. Reference is made to U.S. Pat. No. 5,192,659 to Simons, the teaching of which is incorporated herein by reference for a more detailed description of locus-specific primers.

G. Separation and Detection of DNA Fragments

Once a set of amplified alleles is produced from the multiplex amplification step of the present method, the amplified alleles are evaluated. The evaluation step of this method can be accomplished by any one of a number of different means, the most preferred of which are described below.

Electrophoresis is preferably used to separate the products of the multiplex amplification reaction, more preferably capillary electrophoresis (see, e.g., Buel, Eric et al. (1998), Journal of Forensic Sciences; 43:(1) pp. 164-170) or denaturing polyacrylamide gel electrophoresis (see, e.g., Sambrook, J. et al. (1989) In Molecular Cloning—A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, pp. 13.45-13.57). Gel preparation and electrophoresis procedures and conditions for suitable for use in the evaluating step of the method of this invention are illustrated in the Examples, below. Separation of DNA fragments in a denaturing polyacrylamide gel and in capillary electrophoresis occurs based primarily on fragment size.

Once the amplified alleles are separated, the alleles and any other DNA in the gel or capillary (e.g., DNA size markers or an allelic ladder) can then be visualized and

analyzed. Visualization of the DNA in the gel can be accomplished using any one of a number of prior art techniques, including silver staining or reporters such as radioisotopes, fluorescers, chemiluminescers and enzymes in combination with detectable substrates. However, the 5 preferred method for detection of multiplexes containing thirteen or more loci is fluorescence (see, e.g., Schumm, J. W. et al. in Proceedings from the Eighth International Symposium on Human Identification, (pub. 1998 by Promega Corporation), pp. 78-84; Buel, Eric et al. (1998), supra.), wherein primers for each locus in the multiplexing reaction is followed by detection of the labeled products employing a fluorometric detector. The references cited above, which describe prior art methods of visualizing alleles, are incorporated by reference herein.

The alleles present in the DNA sample are preferably determined by comparison to a size standard such as a DNA marker or a locus-specific allelic ladder to determine the alleles present at each locus within the sample. The most preferred size marker for evaluation of a multiplex ampli- 20 fication containing two or more polymorphic STR loci consists of a combination of allelic ladders for each of the loci being evaluated. See, e.g., Puers, Christoph et al., (1993) Am J. Hum Genet. 53:953-958, Puers, Christoph, et al. (1994) Genomics 23:260-264. See also, U.S. Pat. Nos. 5,599,666; 5,674,686; and 5,783,406 for descriptions of allelic ladders suitable for use in the detection of STR loci, and methods of ladder construction disclosed therein.

Following the construction of allelic ladders for individual loci, these may be mixed and loaded for gel electro- 30 phoresis at the same time as the loading of amplified samples occurs. Each allelic ladder co-migrates with alleles in the sample from the corresponding locus.

The products of the multiplex reactions of the present invention can be evaluated using an internal lane standard, 35 a specialized type of size marker configured to run in the same lane of a polyacrylamide gel or same capillary. The internal lane standard preferably consists of a series of fragments of known length. The internal lane standard more guishable from other dyes in the amplification reaction.

Following construction of the internal lane standard, this standard can also be mixed with amplified sample or allelic ladders and loaded for electrophoresis for comparison of migration in different lanes of gel electrophoresis or different capillaries of capillary electrophoresis. Variation in the migration of the internal lane standard indicates variation in the performance of the separation medium. Quantitation of this difference and correlation with the allelic ladders allows samples.

H. Preferred Detection Technique: Fluorescent Detection

In one of the most preferred embodiments of the method of this invention, fluorescent detection is used to evaluate the amplified alleles in the mixture produced by the multiplex 55 amplification reaction. Below is a brief summary of how that method of detection preferably is practiced.

With the advent of automated fluorescent imaging, faster detection and analysis of multiplex amplification products can be achieved. For fluorescent analysis, one fluorescent labeled primer can be included in the amplification of each locus. Fluorescent labeled primers preferably suited for use in the present invention include the fluorescein-labeled (FL-), carboxy-tetramethylrhodamine-labeled (TMR-), and 5,6-carboxyrhodamine 6G-labeled (R6G) primers, such as 65 are illustrated in the Examples, below. Separation of the amplified fragments produced using such labeled primers is

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achieved preferably by slab gel electrophoresis or capillary electrophoresis. The resulting separated fragments can be analyzed using fluorescence detection equipment such as an ABI PRISM® 310 Genetic Analyzer, an ABI PRISM® 377 DNA Sequencer (Applied Biosystems Division, Perkin Elmer, Foster City, Calif.), or a Hitachi FMBIO® II Fluorescent Scanner (Hitachi Software Engineering America, Ltd. South San Francisco, Calif.).

In summary, the method of this invention is most prefer-10 ably practiced using fluorescent detection as the detection step. In this preferred method of detection, one or both of each pair of primers used in the multiplex amplification reaction has a fluorescent label attached thereto, and as a result, the amplified alleles produced from the amplification 15 reaction are fluorescently labeled. In this most preferred embodiment of the invention, the amplified alleles are subsequently separated by capillary electrophoresis and the separated alleles visualized and analyzed using a fluorescent image analyzer.

Fluorescent detection is preferred over radioactive methods of labeling and detection, because it does not require the use of radioactive materials, and all the regulatory and safety problems which accompany the use of such materials.

Fluorescent detection employing labeled primers is also preferred over other non-radioactive methods of detection, such as silver staining, because fluorescent methods of detection generally reveal fewer amplification artifacts than silver staining. The smaller number of artifacts are due, in part, to the fact that only amplified strands of DNA with labels attached are detected in fluorescent detection, while both strands of every amplified allele of DNA produced from the multiplex amplification reaction is stained and detected using the silver staining method of detection.

The present invention is also directed to kits that utilize the process described above. A basic kit comprises a container having one or more locus-specific primers. Instructions for use optionally may be included.

Other optional kit components may include an allelic preferably is labeled with a fluorescent dye which is distin- 40 ladder directed to each of the specified loci, a sufficient quantity of enzyme for amplification, amplification buffer to facilitate the amplification, loading solution for preparation of the amplified material for electrophoresis, genomic DNA as a template control, a size marker to insure that materials migrate as anticipated in the separation medium, and a protocol and manual to educate the user and to limit error in use. The amounts of the various reagents in the kits also can be varied depending upon a number of factors, such as the optimum sensitivity of the process. It is within the scope of correction in the size determination of alleles in unknown 50 this invention to provide test kits for use in manual applications or test kits for use with automated detectors or analyzers.

EXAMPLES

The following Examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. The Examples are intended to be illustrative, and are not intended in any way to otherwise limit the scope of the claims or protection granted by the patent.

The human genomic DNA samples assayed in the Example below were prepared from blood or tissue culture cells, using a standard procedure described by Miller and Dykes in (Miller, S. et al. (1988) *Nucl. Acids Res.* 16:1215). The isolation and quantification methods described therein are generally known to those skilled in the art and are preferred, but not required, for application of the invention.

Each Example below is an example of the use of the method of this invention, to determine simultaneously the alleles present in at least thirteen loci from one or more DNA samples of human genomic DNA. Each set of loci co-amplified below includes the thirteen short tandem repeat loci identified for use in the CODIS system (i.e., D3S1358, HUMTH01, D21S11, D18S51, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D13S317, D7S820, D16S539, and HUMCSF1PO). Some sets of loci co-amplified below also include one or more additional short tandem repeat loci, such as loci with pentanucleotide repeats (e.g., G475, S159, or C221), and a non-STR locus, Amelogenin.

Table 2 summarizes which set of loci was co-amplified in the multiplex amplification reaction described in each Example below. The table also indicates which primer pair was used to amplify each such locus in each such multiplex reaction. One primer of each primer pair listed on Table 2 was fluorescently labeled prior to being used in the multiplex amplification reaction. In some cases, a different label was used to label primers to different loci, such that the alleles produced using the different primers could be distinguished from one another when detected with a laseractivated fluorescence detection device.

Three different fluorescent labels were used in the Examples below, described as "FL" to indicate fluorescein-labeled, "TMR" to indicate carboxy-tetramethylrhodamine-labeled, and "R6G" to indicate 5,6-carboxyrhodamine 6G in Table 2, below. Table 2 also indicates which primer of each pair of primers used in the multiplex amplification reaction was so labeled in each Example (e.g., "FL-69" means the primer with SEQ ID NO:69 was labeled at its 5' end with fluorescein prior to being used in the multiplex amplification reaction). In the text of each of the Examples, however, the label abbreviation is placed immediately before the SEQ ID NO of the labeled primer used in the amplification reaction described therein (e.g., "FL-SEQ ID NO:2" instead of "FL-2").

TABLE 2

Example	Loci Amplified	Primer Pair: SEQ ID NO's Used	Fluorescent Label(s) Used
1	D3S1358	68,69	FL-69
	HUMTHO1	66,67	FL-66
	D21S11	64,65	FL-65
	D18S51	62,63	FL-62
	HUMvWFA31	76,40	TMR-40
	D8S1179	74,75	TMR-75
	HUMTPOX	72,73	TMR-73
	HUMFIBRA	70,71	TMR-70
	D5S818	84,85	R6G-85
	D13S317	82,83	R6G-83
	D7S820	80,81	R6G-80
	D16S539	29,79	R6G-79
	HUMCSF1PO	77,78	R6G-78
2,3	D3S1358	68,69	FL-69
	HUMTHO1	66,67	FL-66
	D21S11	64,65	FL-65
	D18S51	62,63	FL-62
	G475	88,89	FL-88
	Amelogenin	86,87	TMR-86
	HUMvWFA31	76,40	TMR-40
	D8S1179	74,75	TMR-75
	HUMTPOX	72,73	TMR-73
	HUMFIBRA	70,71	TMR-70
	D5S818	84,85	R6G-85
	D13S317	82,83	R6G-83
	D7S820	80,81	R6G-80
	D16S539	29,79	R6G-79

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TABLE 2-continued

Example	Loci Amplified	Primer Pair: SEQ ID NO's Used	Fluorescent Label(s) Used
	HUMCSF1PO	77,78	R6G-78
	S159	90,91	R6G-91
4	D3S1358	68,69	FL-69
	HUMTHO1	66,67	FL-66
	D21S11	64,65	FL-65
	D18S51	62,63	FL-62
	G475	88,89	FL-88
	Amelogenin	86,87	TMR-86
	HUMvWFA31	76,40	TMR-40
	D8S1179	74,75	TMR-75
	HUMTPOX	72,73	TMR-73
	HUMFIBRA	70,71	TMR-70
	D5S818	84,85	FL-85
	D13S317	82,83	FL-83
	D7S820	80,81	FL-80
	D16S539	29,79	FL-79
	HUMCSF1PO	77,78	FL-78
	S159	90,91	FL-91
5	D3S1358	68,69	FL-69
	HUMTHO1	66,67	FL-66
	D21S11	64,65	FL-65
	D18S51	62,63	FL-62
	G475	88,94	FL-94
	Amelogenin	86,87	TMR-86
	HUMvWFA31	76,40	TMR-40
	D8S1179	74,75	TMR-75
	HUMTPOX	72,73	TMR-73
	HUMFIBRA	70,71	TMR-70
	D5S818	84,85	FL-85
	D13S317	82,83	FL-83
	D7S820	80,81	FL-80
	D16S539	29,79	FL-79
	HUMCSF1PO	77,78	FL-78
	S159	95,96	FL-76
6	D3S1358	69,106	FL-69
O	HUMTHO1	38,103	FL-38
	D21S11	64,65	FL-65
	D18S51	101,102	FL-101
	S159	92,93	FL-93
	Amelogenin	105,87	TMR-105
	HUMvWFA31	76,40	TMR-40
	D8S1179	104,75	TMR-104
	HUMTPOX	72,73	TMR-72
	HUMFIBRA	72,73	TMR-72 TMR-70
	D5S818		FL-85
		84,85	FL-85 FL-4
	D13S317	3,4	
	D7S820	80,81	FL-80
	D16S539	29,97	FL-29
	HUMCSF1PO	77,98	FL-98
	C221	99,100	FL-99

Example 1

Fluorescent Detection of Multiplex Amplification of Loci D3S1358. HUMTH01, D21S11, D18S51, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, and HUMCSF1PO as Detected with the ABI PRISM® 310 Genetic Analyzer

In this Example, a DNA template was amplified simultaneously at the individual loci D3S1358, HUMTH01, D21S11, D18S51, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, and HUMCSF1PO in a single reaction vessel. The PCR amplification was performed in 25 μl of 1×Gold ST*R Buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂, 160 μg/ml BSA and 200 μM each of dATP, dCTP, dGTP and dTTP) using 1 ng template, and 3.25 U AmpliTaq Gold™ DNA Polymerase. A GeneAmp® PCR System 9600 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C.

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for 12 min., then 10 cycles of 94° C. for 30 sec., ramp for 68 sec. to 58° C., hold for 30 sec., ramp 50 sec. to 70° C., hold for 45 sec., followed by 20 cycles of 90° C. for 30 sec., ramp 60 sec. to 58° C., hold for 30 sec., ramp for 50 sec, to 70° C., hold for 45 sec., followed by 1 cycle of 60° C. for 5 30 min

Twenty-six amplification primers were used in combination, including 0.12 µM each D3S1358 primers 1 [SEQ ID NO:68] and 2 [FL-SEQ ID NO:69], 0.08 µM each HUMTH01 primers 1 [FL-SEQ ID NO:66] and 2 [SEQ ID 10 NO:67], 0.3 µM each D21S11 primers 1 [SEQ ID NO:64] and 2 [FL-SEQ ID NO:65], 0.2 μ M each D18S51 primers 1 [FL-SEG ID NO:62] and 2 [SEQ ID NO:63], 1.1 μ M each HUMvWFA31 primers 1 [SEQ ID NO:76] and 2 [TMR-SEQ ID NO:40], 1.8 μ M each D8S1179 primers 1 [SEQ ID 15] NO:74] and 2 [TMR-SEQ ID NO:75], $0.6 \mu M$ each HUMT-POX primers 1 [SEQ ID NO:72] and 2 [TMR-SEQ ID NO:73], 2.4 μ M each HUMFIBRA primers 1 [TMR-SEQ ID NO:70] and 2 [SEQ ID NO:71], 0.2 µM each D5S818 primers 1 [SEQ ID NO:84] and 2 [R6G-SEQ ID NO:85], 0.1 20 μM each D13S317 primers 1 [SEQ ID NO:82] and 2 [R6G-SEQ ID NO:83], 0.2 µM each D7S820 primers 1 [R6G-SEQ ID NO:80]and 2 [SEQ ID NO:81], 0.15 µM each D16S539 primers 1 [SEQ ID NO:29] and 2 [R6G-SEQ ID NO:79], 0.2 μ M each HUMCSF1PO primers 1 [SEQ ID 25] NO:77] and 2 [R6G-SEQ ID NO:78]

Amplified products were separated using an ABI PRISM® 310 Genetic Analyzer. DNA samples were mixed with 24 μ l of a loading solution (deionized formamide) and 1.0 μ l of an internal lane size standard, denatured at 95° C. for 3 min., and chilled on ice prior to injection. Separation was carried out using Performance Optimized Polymer 4 (POP-4)(Perkin Elmer Biosystems, Foster City, Calif.) in a 47 cm×50 μ m capillary. The manufacturer's GeneScan® run module GS STR POP4 (Id.) (1 ml) A was used. Conditions for the electrophoresis were a 5 second injection, injection kV was 15.0, run kV was 15.0, run temperature was 60° C., run time was 28 minutes and virtual filter A was used.

FIG. 1A is a printout of results of scanning the amplified fragments of each locus separated and detected with the ABI PRISM® 310 Genetic Analyzer, as described above. FIG. 1A shows amplification products of a DNA sample simultaneously co-amplified for the loci D3S1358, HUMTH01, D21S11, D18S51, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, and HUMCSF1PO. Peaks shown in Panel A are labeled with fluorescein, peaks shown in Panel B are labeled with carboxy-tetramethylrhodamine, and peaks shown in Panel C are labeled with 5,6carboxyrhodamine 6G.

FIG. 1B is a printout of the results of scanning a sample prepared in the same way as the sample scanned in FIG. 1A, except that no DNA template was used in the amplification reaction. Peaks in this figure are background products resulting from dye conjugation and purification procedures and from undefined causes.

Example 2

Fluorescent Detection of Multiplex Amplification of Loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and S159 as detected with the ABI PRISM® 310 Genetic Analyzer

In this Example, a DNA template was amplified simultaneously at the individual loci D3S1358, HUMTH01,

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D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and S159 in a single reaction vessel. The PCR amplification was performed in 25 μl of 1×Gold ST*R Buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂, 160 μg/ml BSA and 200 μM each of dATP, dCTP, dGTP and dTTP) using 1 ng template, and 4 U AmpliTaq GoldTM DNA Polymerase. A GeneAmp® PCR System 9600 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 12 min., then 10 cycles of 94° C. for 30 sec., ramp for 68 sec. to 58° C., hold for 30 sec., ramp 50 sec. to 70° C., hold for 45 sec., followed by 20 cycles of 90° C. for 30 sec., ramp 60 sec. to 58° C., hold for 30 sec., ramp for 50 sec, to 70° C., hold for 45 sec., followed by 1 cycle of 60° C. for 30 min.

Thirty-two amplification primers were used in combination, including 0.12 µM each D3S1358 primers 1 [SEQ ID NO:68] and 2 [FL-SEQ ID NO:69], 0.08 μ M each HUMTH01 primers 1 [FL-SEQ ID NO:66] and 2 [SEQ ID NO:67], 0.3 μM each D21S11 primers 1 [SEQ ID NO:64] and 2 [FL-SEQ ID NO:65], 0.2 µM each D18S51 primers 1 [FL-SEQ ID NO:62] and 2 [SEQ ID NO:63], 0.24 μ M each G475 primers 1 [FL-SEQ ID NO:88] and 2 [SEQ ID NO:89], 0.6 μM each Amelogenin primers 1 [TMR-SEQ ID NO:86] and 2 [SEQ ID NO:87], 1.1 μ M each HUMvWFA31 primers 1 [SEQ ID NO:76] and 2 [TMR-SEQ ID NO:40], 1.8 μ M each D8S1179 primers 1 [SEQ ID NO:74] and 2 [TMR-SEQ ID NO:75], 0.6 µM each HUMTPOX primers 1 [SEQ ID NO:72] and 2 [TMR-SEQ ID NO:73], $2.4 \mu M$ each HUMFIBRA primers 1 [TMR-SEQ ID NO:70] and 2 [SEQ ID NO:71], 0.2μ M each D5S818 primers 1 [SEQ ID NO:84] and 2 [R6G-SEQ ID NO:85], 0.1 μM each D13S317 primers 1 [SEQ ID NO:82] and 2 [R6G-SEQ ID NO:83], 0.2 μ M each D7S820 primers 1 [R6G-SEQ ID NO:80] and 2 [SEQ ID NO:81], 0.15 μ M each D16S539 primers 1 [SEQ ID NO:29] and 2 [R6G-SEQ ID NO:79], 0.2 µM each HUMCSF1PO primers 1 [SEQ ID NO:77] and 2 [R6G-SEQ ID NO:78] 0.1 μ M each S159 primers 1 [SEQ ID NO:90] $_{\rm 40}~$ and 2 [R6G-SEQ ID NO:91].

Amplified products were separated using an ABI PRISM® 310 Genetic Analyzer. DNA samples were mixed with 24 μl of a loading solution (deionized formamide) and 1.0 μl of an internal lane size standard, denatured at 95° C. for 3 min., and chilled on ice prior to injection. Separation was carried out using Performance Optimized Polymer 4 (POP-4) (Perkin Elmer Biosystems, Foster City, Calif.) in a 47 cm×50 μm capillary. The manufacturer's GeneScan® run module GS STR POP4 (Id.)(1 ml) A was used. Conditions for the electrophoresis were a 5 second injection, injection kV was 15.0, run kV was 15.0, run temperature was 60° C., run time was 28 minutes and virtual filter A was used.

FIG. 2A is a printout of results of scanning the amplified fragments of each locus separated and detected with the ABI PRISM® 310 Genetic Analyzer, as described above. FIG. 2A shows amplification products of a DNA sample simultaneously co-amplified for the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO, and S159. Peaks shown in Panel A are labeled with fluorescein, peaks shown in Panel B are labeled with carboxy-tetramethylrhodamine, and peaks shown in Panel C are labeled with 5,6carboxyrhodamine 6G.

FIG. 2B is a printout of the results of scanning a sample prepared in the same way as the sample scanned in FIG. 2A, except that no DNA template was used in the amplification

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reaction. Peaks in this figure are background products resulting from dye conjugation and purification procedures and from undefined causes.

Example 3

Fluorescent Detection of Multiplex Amplification of Loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO, and S159 as detected with the ABI PRISM® 377 DNA Sequencer

In this Example, a DNA template was amplified as in Example 2. Amplified products were separated using an ABI PRISM® 377 DNA Sequencer. This was carried out using a 0.2 mm thick, 5% Long Ranger Acrylamide (FMC BioProducts, Rockland, Me.), 7M urea gel. DNA samples were mixed with 1.5 μ l of a loading solution (88.25% formamide, 4.1 mM EDTA, 15 mg/ml Blue Dextran) and 0.5 μ l of an internal lane size standard, denatured at 95° C. for 2 min., and chilled on ice prior to loading. Electrophoresis was carried out using the manufacturer's GeneScan® modules for Prerun (PR GS 36A-2400) and Run (GS 36A-2400). Run time was 3 hours and virtual filter A was used.

FIG. 3A is a printout of results of scanning the amplified fragments of each locus separated and detected with the ABI PRISM® 377 DNA Sequencer, as described above. FIG. 3A shows amplification products of a DNA sample simultaneously co-amplified for the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO, and S159. Peaks shown in Panel A are labeled with fluorescein, peaks shown in Panel B are labeled with carboxy-tetramethylrhodamine, and peaks shown in Panel C are labeled with 5,6carboxyrhodamine 6G.

FIG. 3B is a printout of the results of scanning a sample prepared in the same way as the sample scanned in FIG. 3A, except that no DNA template was used in the amplification reaction. Peaks in this figure are background products resulting from dye conjugation and purification procedures and from undefined causes.

Example 4

Fluorescent Detection of Multiplex Amplification of Loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO, and S159 as Detected with the Hitachi FMBIO® II Fluorescent Scanner

In this example, two DNA templates were each amplified simultaneously at each of three different locus combinations selected from the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and S159. Amplification of each locus combination included 5 ng template in a single reaction vessel containing 25 µl of 1×Gold ST*R Buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂, 160 µg/ml BSA and 200 µM each of dATP, dCTP, dGTP and dTTP).

A GeneAmp® PCR System 9600 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification

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protocol: 96° C. for 12 min., then 10 cycles of 94° C. for 30 sec., ramp for 68 sec. to 58° C., hold for 30 sec., ramp 50 sec. to 70° C., hold for 45 sec., followed by 22 cycles of 90° C. for 30 sec., ramp 60 sec. to 58° C., hold for 30 sec., ramp for 50 sec, to 70° C., hold for 45 sec., followed by 1 cycle of 60° C. for 30 min.

Thirty-two amplification primers were used in the following concentrations, including 0.225 μ M each D3S1358 primers 1 [SEQ ID NO:68] and 2 [FL-SEQ ID NO:69], 0.2 $_{10}~\mu M$ each HUMTH01 primers 1 [FL-SEQ ID NO:66] and 2 [SEQ ID NO:67], 1.0 μ M each D21S11 primers 1 [SEQ ID NO:64] and 2 [FL-SEQ ID NO:65], 1.0 μM each D18S51 primers 1 [FL-SEQ ID NO:62] and 2 [SEQ ID NO:63], 2.8 μM each G475 primers 1 [FL-SEQ ID NO:88] and 2 [SEQ ID NO:89], $0.2 \mu M$ each Amelogenin primers 1 [TMR-SEQ ID NO:86] and 2 [SEQ ID NO:87], 0.3 μM each HUMvWFA31 primers 1 [SEQ ID NO:76] and 2 [TMR-SEQ ID NO:40], 1,5 μ M each D8S1179 primers 1 [SEQ ID NO:74] and 2 [TMR-SEQ ID NO:75], 0.2 μ M each HUMTPOX primers 1 [SEQ ID NO:72] and 2 [TMR-SEQ ID NO:73], 2.0 µM each HUMFIBRA primers 1 [TMR-SEQ ID NO:70] and 2 [SEQ ID NO:71], 0.55 μ M each D5S818 primers 1 [SEQ ID NO:84] and 2 [FL-SEQ ID NO:85], 1.1 μ M each D13S317 primers 1 [SEQ ID NO:82] and 2 [FL-SEQ ID NO:83], 1.7 μ M each D7S820 primers 1 [FL-SEQ ID NO:80] and 2 [SEQ ID NO:81], 3.3 μM each D16S539 primers 1 [SEQ ID NO:29] and 2 [FL-SEQ ID NO:79], 0.5 μ M each HUMCSF1PO primers 1 [SEQ ID NO:77] and 2 [FL-SEQ ID NO:78], 2.0 µM each S159 primers 1 [SEQ ID NO:90] and 2 [FL-SEQ ID NO:91].

In the first locus combination, each template was amplified using 2.5 U of AmpliTaq Gold™ DNA Polymerase and primers for each locus used in the concentrations described above for the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, and HUMFIBRA. In the second locus combination, all thirty-two primers, above, at the described concentrations, and 4 U of AmpliTaq Gold™ DNA Polymerase were used to amplify DNA templates at all sixteen loci, D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and S159 in a single reaction vessel. In the third combination, each template was amplified using 1.5 U of AmpliTaq 45 Gold™ DNA Polymerase and primers for each locus used in the concentrations described above for the loci D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and S159.

Amplification products were separated by electrophoresis through a 0.4 mm thick 4% denaturing polyacrylamide gel 50 (19:1 ratio of acrylamide to bis-acrylamide) which contained 7 M urea (Sambrook et al., (1989)), and which was chemically cross-linked to 2 glass plates (Kobayashi, Y. (1988), BRL Focus 10: 73–74). DNA samples were mixed with 3.5 μ l of a loading solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue) and $0.5 \mu l$ of an internal lane size standard, denatured at 95° C. for 2 min., and chilled on ice prior to loading. The separated products were visualized by detection of the fluorescent signals using the Hitachi FMIBO® II fluorescent scanner (Hitachi Software Engineering America, Ltd. South San Francisco, Calif.). Band pass filters at 505 nm and 585 nm, respectively, were used for the detection of fluorescein-labeled loci and carboxytetramethylrhodamine-labeled loci, respectively. A band pass filter of 650 nm was used for detection of the internal 65 lane standard (size standard data, not shown).

Reference is made to FIGS. 4A and 4B, which display the fragments resulting from each amplification reaction. FIG.

4A shows the results from the 505 nm scan (Fluorescein channel) and FIG. 4B shows the results from the 585 nm scan (carboxy-tetramethylrhodamine channel) of the same lanes of the polyacrylamide gel. For each DNA template, lane 1 shows the results of the DNA sample which has been 5 simultaneously co-amplified for the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, and HUMFIBRA. Lane 2 shows the results of the DNA sample simultaneously co-amplified for the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D13S317, D7S820, D16S539, HUMCSF1PO, and S159. Lane 3 shows the results of the DNA sample simultaneously co-amplified for the loci D5S818, D13S317, D7S820, D16S539, 15 HUMCSF1PO, and S159.

Example 5

Fluorescent Detection of Multiplex Amplification of Loci D3S1358, HUMTH01, D2IS11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO, and S159 as detected with the Hitachi FMBIO® II Fluorescent Scanner

In this example, two DNA templates were each amplified simultaneously at each of two different locus combinations selected from the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, 30 HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and S159. Amplification of each locus combination included 5 ng template in a single reaction vessel containing 25 μ l of 1×Gold ST*R Buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25° C.), 0.1% Triton 35 X-100, 1.5 mM MgCl₂, 160 μ g/ml BSA and 200 μ M each of dATP, dCTP, dGTP and dTTP).

A GeneAmp® PCR System 9600 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 12 min., then 10 cycles of 94° C. for 30 40 sec., ramp for 68 sec. to 58° C., hold for 30 sec., ramp 50 sec. to 70° C., hold for 45 sec., followed by 22 cycles of 90° C. for 30 sec., ramp 60 sec. to 58° C., hold for 30 sec., ramp for 50 sec, to 70° C., hold for 45 sec., followed by 1 cycle of 60° C. for 30 min.

Thirty-two amplification primers were used in the following concentrations, including 0.225 µM each D3S1358 primers 1 [SEQ ID NO:68] and 2 [FL-SEQ ID NO:69], 0.2 µM each HUMTH01 primers 1 [FL-SEQ ID NO;66] and 2 NO:64] and 2 [FL-SEQ ID NO:65], 1.0 M each D18S51 primers 1 [FL-SEQ ID NO:62] and 2 [SEQ ID NO:63], 2.8 μM each G475 primers 1 [SEQ ID NO:88] and 2 [FL-SEQ ID NO:94], 0.2 μM each Amelogenin primers 1 [TMR-SEQ ID NO:86] and 2 [SEQ ID NO:87], 0.3 μ M each HUMvWFA31 primers 1 [SEQ ID NO:76] and 2 [TMR-SEQ ID NO:40], 1.5 μ M each D8S1179 primers 1 [SEQ ID NO:74] and 2 [TMR-SEQ ID NO:75], 0.2 µM each HUMTPOX primers 1 [SEQ ID NO:72] and 2 [TMR-SEQ ID NO:73], 2.0 μM each HUMFIBRA primers 1 [TMR-SEQ ID NO:70] and 2 [SEQ ID NO:71], 0.55 μ M each D5S818 primers 1 [SEQ ID NO:84] and 2 [FL-SEQ ID NO:85], 1.1 μ M each D13S317 primers 1 [SEQ ID NO:82] and 2 [FL-SEQ ID NO:83], 1.7 μ M each D7S820 primers 1 [FL-SEQ ID NO:80] and 2 [SEQ ID NO:81], 3.3 μ M each D16S539 primers 1 [SEQ ID NO:29] and 2 [FL-SEQ ID NO:79], 0.5 μM primers 1 [TMR-SEQ ID NO:104] and 2 [SEQ ID

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NO:75], 0.75 μ M each HUMTPOX primers 1 [TMR-SEQ ID NO:72] and 2 [SEQ ID NO:73], 1.5 μ M each HUMFI-BRA primers 1 [TMR-SEQ ID NO:70] and 2 [SEQ ID NO:107], 0.55 μ M each D5S818 primers 1 [SEQ ID NO:84] and 2 [FL-SEQ ID NO:85], 1.1 μ M each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO-4], 1.7 μ M each D7S820 primers 1 [FL-SEQ ID NO:80] and 2 [SEQ ID NO:81], 3.3 μ M each D16S39 primers 1 [FL-SEQ ID NO:29] and 2 [SEQ ID NO:97], 0.25 μ M each HUMCSF1PO primers 1 [SEQ ID NO:77] and 2 [FL-SEQ ID NO:98], 1.0 μ M each C221 primers 1 [FL-SEQ ID NO:99] and 2 [SEQ ID NO:100].

In the first locus combination, each template was amplified using 2.5 U of AmpliTaq Gold™ DNA Polymerase and primers for each locus used in the concentrations described above for the loci D3S1358, HUMTH01, D21S11, D18S51, S159, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, and HUMFIBRA. In the second locus combination, all thirty-two primers, above, at the described concentrations and 4 U of AmpliTaq Gold™ DNA Polymerase were used to amplify DNA templates at all sixteen loci, D3S1358, HUMTH01, D21S11, D18S51, S159, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and C221 in a single reaction vessel. In the third combination, each template was amplified using 1.5 U of AmpliTaq Gold™ DNA Polymerase and primers for each locus used in the concentrations described above for the loci D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and C221.

The amplification products were separated and detected as described in Example 4, except that each sample of amplification products was diluted 1:4 in 1×STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂, and 200 μ M each of dATP, dCTP, dGTP and dTTP). The diluted amplification products (2.5 μ l) were mixed with 2.5 μ l of a loading solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue), without an internal lane standard, denatured at 95° C. for 2 min., and chilled on ice prior to loading.

Example 6

Fluorescent Detection of Multiplex Amplification of Loci D3S1358, HUMTH01, D21S11, D18S51, S159, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D 13S317, D16S539, HUMCSF1PO, and C221 as Detected with the Hitachi FMBIO® II Fluorescent Scanner

in this example, two DNA templates were each amplified simultaneously at each of three different locus combinations selected from the loci D3S1358, HUMTH01, D21S11, primers 1 [FL-SEQ ID NO:63], 2.8 μM each G475 primers 1 [SEQ ID NO:88] and 2 [FL-SEQ ID NO:88] and 2 [FL-SEQ ID NO:94], 0.2 μM each Amelogenin primers 1 [TMR-SEQ ID NO:86] and 2 [SEQ ID NO:76] and 2 [TMR-SEQ ID NO:76] and 2 [TMR-SEQ ID NO:76] and 2 [TMR-SEQ ID NO:75], 0.2 μM each HUMTPOX and C221. Amplification of each solution included 10 ng template in a single reaction vessel containing 25 μl of 1×Gold ST*R Buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂, 160 μg/ml BSA and 200 μM each of dATP, dCTP, dGTP and dTTP).

A GeneAmp® PCR System 9600 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 12 min., then 10 cycles of 94° C. for 30 sec., ramp for 68 sec. to 60° C., hold for 30 sec., ramp 50 sec. to 70° C., hold for 45 sec., followed by 20 cycles of 90° C. for 30 sec., ramp 60 sec. to 60° C., hold for 30 sec., ramp for 50 sec, to 70° C., hold for 45 sec., followed by 1 cycle of 60° C. for 30 min.

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Thirty-two amplification primers were used in the following concentrations, including 0.75 μ M each D3S1358 primers 1 [SEQ ID NO:106] and 2 [FL-SEQ ID NO:69], 0.3 μ M each HUMTH01 primers 1 [FL-SEQ ID NO:38] and 2 [SEQ ID NO:103], 2.0 μ M each D21S11 primers 1 [SEQ ID SO:64] and 2 [FL-SEQ ID NO:65], 0.3 μ M each D18S51 primers 1 [FL-SEQ ID NO:101] and 2 [SEQ ID NO:102], 2.0 μ M each S159 primers 1 [SEQ ID NO:92] and 2 [FL-SEQ ID NO:93], 0.15 μ M each Amelogenin primers 1 [TMR-SEQ ID NO:105] and 2 [SEQ ID NO:87], 1.0 μ M 10 each HUMvWFA31 primers 1 [SEQ ID NO:76] and 2 [TMR-SEQ ID NO:40], 1.25 μ M each D8S1179 each HUMCSF1PO primers 1 [SEQ ID NO:77] and 2 [FL-SEQ ID NO:78], 2.0 μ M each S159 primers 1 [SEQ ID NO:95] and 2 [FL-SEQ ID NO:96].

In the first locus combination, each template was amplified using 2.5 U of AmpliTaq Gold™ DNA Polymerase and primers for each locus used in the concentrations described above for the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, ²⁰ and HUMFIBRA. In the second locus combination, all thirty-two primers, above, at the described concentrations, and 4 U of AmpliTaq Gold™ DNA Polymerase were used to amplify DNA templates at all sixteen loci, D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, ²⁵ HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and S159 in a single reaction vessel.

The separation and visualization of amplified products were as described in Example 4.

Reference is made to FIG. 5A and 5B, which display the fragments resulting from each amplification reaction. FIG.

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5A shows the results from the 505 nm scan (Fluorescein channel) and FIG. 5B shows the results from the 585 nm scan (carboxy-tetramethylrhodamine channel) of the same lanes of the polyacrylamide gel. For each template, lane 1 shows the results of the DNA sample simultaneously co-amplified for the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, and HUMFIBRA and lane 2 shows the results of the DNA sample simultaneously co-amplified for the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D13S317, D7S820, D16S539, HUMCSF1PO, and S159.

Reference is made to FIGS. 6A and 6B, which display the fragments resulting each amplification reaction. FIG. 6A shows the results from the 505 nm scan (Fluorescein channel) and FIG. 6B shows the results from the 585 nm scan (carboxy-tetramethylrhodamine channel) of the same lanes of the polyacrylamide gel. For each DNA template, lane 1 shows the results of the DNA sample simultaneously co-amplified for the loci D3S1358, HUMTH01, D21S11, D18S51, S159, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, and HUMFIBRA. Lane 2 shows the results of the DNA sample simultaneously co-amplified for the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D13S317, D7S820, D16S539, HUMCSF1PO, and C221. Lane 3 shows the results of the DNA sample simultaneously co-amplified for the loci D5S818, D13S317, D7S820, D16S539, HUMCSF1PO, and C221.

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aaaaagacac gccacaggct aagagaaagt acttctaatc acatatctaa

750

57 58

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cagaactagc aaaggaaaag agaagtgaat gtatc	335	

What is claimed is:

1. A method of simultaneously determining the alleles present in a set of short tandem repeat loci from one or more DNA samples, comprising:

- (a) obtaining at least one DNA sample to be analyzed,
- (b) selecting a set of loci of the DNA sample, comprising D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, and HUMvWFA31,
- (c) co-amplifying the loci in the set in a multiplex amplification reaction, wherein the product of the reac-
- tion is a mixture of amplified alleles from each of the co-amplified loci in the set; and
- (d) evaluating the amplified alleles in the mixture to determine the alleles present at each of the loci analyzed in the set within the DNA sample.
- 2. The method of claim 1, wherein at least four of the short tandem repeat loci are selected from the group consisting of:
 D5S818, D7S820, D13S317, D16S539, D18S51, D21S11, D3S1358, D8S1179, HUMFIBRA, HUMCSF1PO, HUMTPOX, HUMTH01, and HUMvWFA31.

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- 3. The method of claim 1, wherein at least one of the short tandem repeat loci in the set of loci selected in step (b) is a pentanucleotide tandem repeat locus.
- 4. The method of claim 1, wherein the set of loci selected in step (b) further comprises a locus which can be used to 5 identify the gender of at least one source of the DNA provided in step (a).
- 5. The method of claim 2, wherein the multiplex amplification reaction is done using at least one oligonucleotide primer having a sequence selected from at least one of each 10 comprising: of the groups of primers consisting of:

 (a) obtain
 - SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:80 and SEQ ID NO:81, when one of the loci in the set is D7S820;
 - SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:82, and SEQ ID NO:83, when one of the loci in the set is D13S317; 15
 - SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:84, and SEQ ID NO:85, when one of the loci in the set is D5S818;
 - SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:58, SEQ ID NO:79, and SEQ ID NO:97, when one of the loci in the set is D16S539;
 - SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:77, SEQ ID NO:78, and SEQ ID NO:98, when one of the loci in the set is HUMCSF1PO;
 - SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:72 and SEQ 25 ID NO:73, when one of the loci in the set is HUMT-POX:
 - SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:66, SEQ ID NO:67, and SEQ ID NO:103, when one of the loci in the set is HUMTH01;
 - SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:59, SEQ ID NO:60, and SEQ ID NO:76 when one of the loci in the set is HUMvWFA31;
 - SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:101, and SEQ ID NO:102, when one of the loci in the set is D18S51;
 - SEQ ID NO:64 and SEQ ID NO:65, when one of the loci in the set is D21S11;
 - SEQ ID NO:68, SEQ ID NO:69, and SEQ ID NO:106, $_{\rm 40}$ when one of the loci in the set is D3S1358;
 - SEQ ID NO:70, SEQ ID NO:71, and SEQ ID NO:107, when one of the loci in the set is HUMFIBRA; and
 - SEQ ID NO:74, SEQ ID NO:75, and SEQ ID NO:104, when one of the loci in the set is D8S1179.
- 6. The method of claim 1, wherein the amplified alleles are separated prior to evaluating in step (d), using a separation means selected from the group consisting of polyacrylamide gel electrophoresis and capillary gel electrophoresis
- 7. The method of claim 1, wherein the multiplex amplification reaction is done using pairs of oligonucleotide primers flanking the loci analyzed.
- **8**. The method of claim **7**, wherein the set of loci is co-amplified using a polymerase chain reaction.
- 9. The method of claim 7, wherein each of the loci co-amplified in the multiplex reaction in step (b) is co-amplified using a pair of primers which flank the locus, wherein at least one primer of each pair has a fluorescent label covalently attached thereto.
- 10. The method of claim 9, wherein at least three of the labeled primers have different fluorescent labels covalently attached thereto.
- 11. The method of claim 1 wherein the at least one DNA sample to be analyzed is prepared from human tissue, 65 wherein the human tissue is selected from the group of human tissue consisting of blood, semen, vaginal cells, hair,

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saliva, urine, amniotic fluid containing placental cells or fetal cells, and mixtures of any of the tissues listed above.

- 12. The method of claim 1, wherein the amplified alleles are evaluated by comparing the amplified alleles to a size standard, wherein the size standard is selected from the group of size standards consisting of a DNA marker and a locus-specific allelic ladder.
- 13. A method of simultaneously identifying the alleles present in a set of loci of from one or more DNA samples, comprising:
 - (a) obtaining at least one DNA sample to be analyzed;
 - (b) selecting a set of loci of the DNA sample, comprising short tandem repeat loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, and HUMvWFA31;
 - (c) co-amplifying the loci in the set in a multiplex amplification reaction, wherein the product of the reaction is a mixture of amplified alleles from each of the co-amplified loci in the set; and
 - (d) evaluating the amplified alleles in the mixture to determine the alleles present at each of the loci analyzed in the set within the DNA sample.
- 14. The method of claim 13, wherein the multiplex amplification reaction is done using at least one primer for at least one locus in the set of loci selected in step (b), wherein the primer has a sequence selected from each of the groups of primer sequences consisting of:
- SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:101, and SEQ ID NO:102, when one of the loci in the set is D18S51;
- SEQ ID NO:64 and SEQ ID NO:65, for the locus D21S11:
- SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:66, SEQ ID NO:67, and SEQ ID NO:103, for the locus HUMTH01;
- SEQ ID NO:68, SEQ ID NO:69, and SEQ ID NO:106, for the locus D3S1358:
- SEQ ID NO:70, SEQ ID NO:71, and SEQ ID NO:107, for the locus HUMFIBRA;
- SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:72, and SEQ ID NO:73, for the locus HUMTPOX;
- SEQ ID NO:74, SEQ ID NO:75, and SEQ ID NO:104, for the locus D8S1179;
- SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:59, SEQ ID NO:60, and SEQ ID NO:76, for the locus HUMvWFA31;
- SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:77, SEQ ID NO:78, and SEQ ID NO:98, for the locus HUMCSF1PO;
- SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:58, SEQ ID NO:79, and SEQ ID NO:97, for the locus D16S539;
- SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:80, and SEQ ID NO:81, for the locus D7S820;
- SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:82, and SEQ ID NO:83, for the locus D13S317; and
- SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:84, and SEQ ID NO:85, for the locus D5S818.
- 15. The method of claim 13, wherein the multiplex amplification reaction is a polymerase chain reaction.
- 16. The method of claim 13, wherein the amplified alleles are evaluated by comparing the amplified alleles to a size standard, wherein the size standard is selected from the group of size standards consisting of a DNA marker and a locus-specific allelic ladder.

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- 17. The method of claim 13 wherein the at least one DNA sample to be analyzed is prepared from human tissue, wherein the human tissue is selected from the group of human tissue consisting of blood, semen, vaginal cells, hair, saliva, urine, bone, buccal sample, amniotic fluid containing 5 placental cells or fetal cells, and mixtures of any of the tissues listed above.
- 18. A kit for simultaneously analyzing a set of loci of genomic DNA comprising oligonucleotide primers for co-amplifying a set of loci of the genomic DNA to be 10 analyzed, wherein the set of loci comprises short tandem repeat loci which can be co-amplified, the primers are in one or more containers, the genomic DNA is human genomic DNA, and the loci comprise D3S1358, D5S818, D7S820, D8S1179, D1S317, D16S539, D18S51, D21S11, 15 attached thereto, and at least two of the oligonucleotide HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, AND HUMvWFA31.
- 19. The kit of claim 18, wherein all of the oligonucleotide primers in the kit are in one container.
- 20. The kit of claim 18, wherein at least one of the primers 20 for co-amplifying a locus in the set of loci has a sequence selected from one of the groups of primer sequences con-
 - SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:101, and SEQ ID NO:102, for D18S51;
 - SEQ ID NO:64 and SEQ ID NO:65, for D21S11,
 - SEO ID NO:66, SEQ ID NO:67, SEQ ID NO:38, and SEQ ID NO:103, for HUMTH01
 - SEQ ID NO:68, SEQ ID NO:69, and SEQ ID NO:106, for 30 D3S1358
 - SEQ ID NO:70, SEQ ID NO:71, and SEQ ID NO:107, for **HUMFIBRA**
 - SEQ ID NO:72 and SEQ ID NO:73, for HUMTPOX
 - SEQ ID NO:74, SEQ ID NO:75, and SEQ ID NO:104, for 35 D8S1179
 - SEQ ID NO:76 and SEQ ID NO:40, for HUMvWFA31 SEQ ID NO:77, SEQ ID NO:78, and SEQ ID NO:98, for HUMCSF1PO

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- SEQ ID NO:29, SEQ ID NO:79, and SEQ ID NO:97, for D16S539
- SEQ ID NO:80 and SEQ ID NO:81, for D7S820
- SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:82, and SEQ ID NO:83, for D13S317
- SEQ ID NO:84 and SEQ ID NO:85, for D5S818.
- 21. The kit of claim 18, further comprising reagents for at least one multiplex amplification reaction.
- 22. The kit of claim 18, further comprising a container having an allelic ladder.
- 23. The kit of claim 22, wherein each rung of the allelic ladder and at least one oligonucleotide primer for each of the loci in the set each have a fluorescent label covalently primers have a different fluorescent label covalently attached thereto than other primers in the container.
- 24. A method of simultaneously determining the alleles present in a set of loci from one or more DNA samples, comprising:
 - (a) obtaining at least one DNA sample to be analyzed;
 - (b) selecting a set of loci of the at least one DNA sample, comprising at least thirteen short tandem repeat loci which can be co-amplified, wherein at least four of the at least thirteen short tandem repeat loci are selected from the group comprising: D5S818, D7S820, D13S317, D16S539, D18S51, D21S11, D3S1358, D8S1179, HUMFIBRA, HUMCSF1PO, HUMTPOX, HUMTH01, and HUMvWFA31;
 - (c) co-amplifying the loci in the set in a multiplex amplification reaction, wherein the product of the reaction is a mixture of amplified alleles from each of the co-amplified loci in the set; and
 - (d) evaluating the amplified alleles in the mixture to determine the alleles present at each of the loci analyzed in the set within the DNA sample.

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TAB 9

US007008771B1

(12) United States Patent

Schumm et al.

US 7,008,771 B1 (10) Patent No.:

Mar. 7, 2006 (45) Date of Patent:

(54) MULTIPLEX AMPLIFICATION OF SHORT TANDEM REPEAT LOCI

(75) Inventors: James W. Schumm, Madison, WI (US); Cynthia J. Sprecher, Madison,

WI (US)

Assignee: Promega Corporation, Madison, WI

Notice: Subject to any disclaimer, the term of this

patent is extended or adjusted under 35

U.S.C. 154(b) by 389 days.

(21) Appl. No.: 10/236,577

(22) Filed: Sep. 6, 2002

Related U.S. Application Data

Division of application No. 09/199,542, filed on Nov. 25, 1998, now Pat. No. 6,479,235, which is a continuation-in-part of application No. 08/632,575, filed on Apr. 15, 1996, now Pat. No. 5,843,660, which is a continuation-in-part of application No. 08/316,544, filed on Sep. 30, 1994, now abandoned.

(51) Int. Cl. C07H 21/04 (2006.01)C12Q 1/68 (2006.01)

536/23.1; 536/24.3

Field of Classification Search 435/6, 435/91.1, 91.2; 536/23.1, 24.3

See application file for complete search history.

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ABSTRACT

Methods and materials are disclosed for use in simultaneously amplifying at least thirteen loci of genomic DNA in a single multiplex reaction, as are methods and materials for use in the analysis of the products of such reactions. Included in the present invention are materials and methods for the simultaneous amplification of at least thirteen short tandem repeat loci, including specific materials and methods for the analysis of thirteen such loci specifically selected by the United States Federal Bureau of Investigation as core loci for use in the Combined DNA Index System (CODIS) database.

6 Claims, 12 Drawing Sheets

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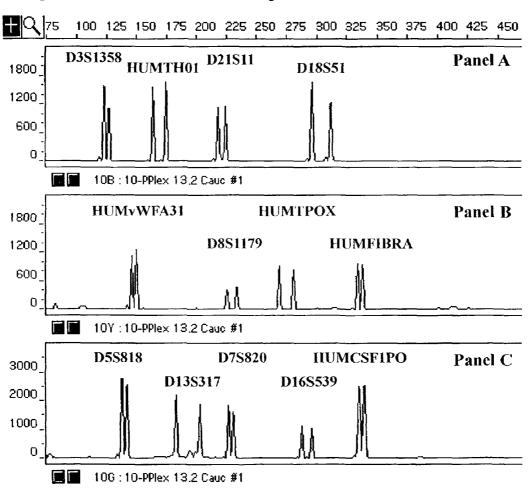
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FIG. 1A

Amplification with DNA template

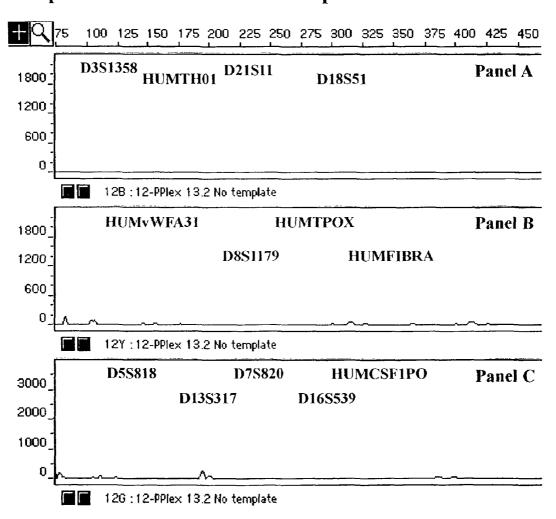


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FIG. 1B

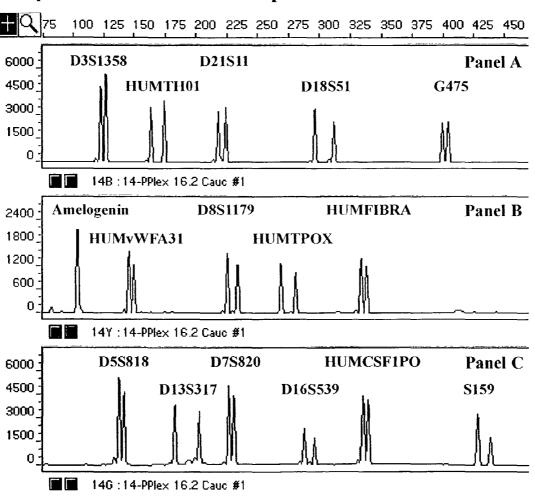
Amplification with NO DNA template



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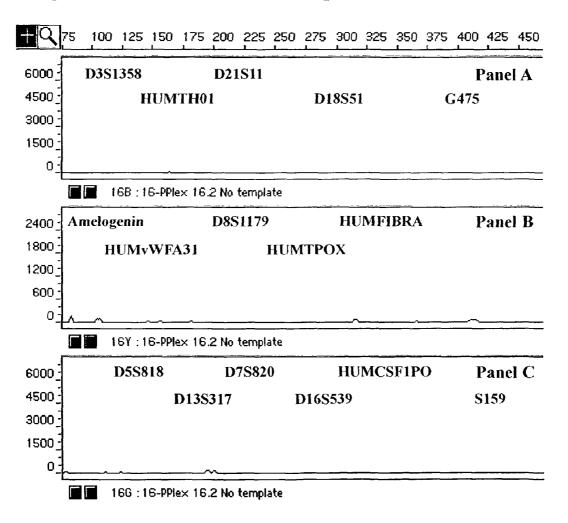
FIG. 2A
Amplification with DNA template



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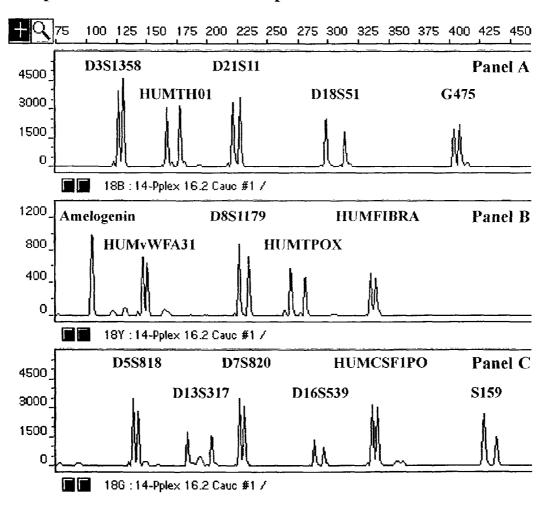
FIG. 2B
Amplification with NO DNA template



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FIG. 3A
Amplification with DNA template

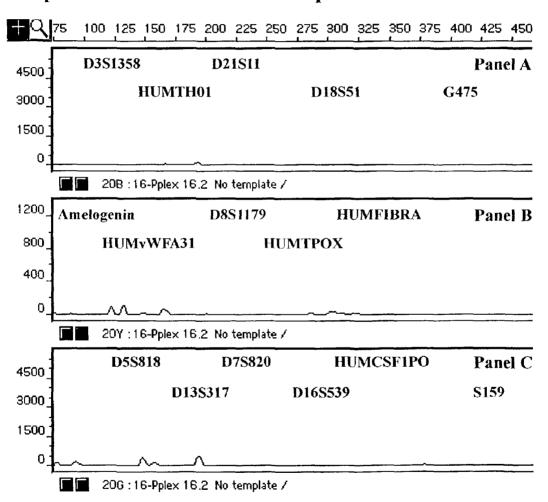


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FIG. 3B

Amplification with NO DNA template



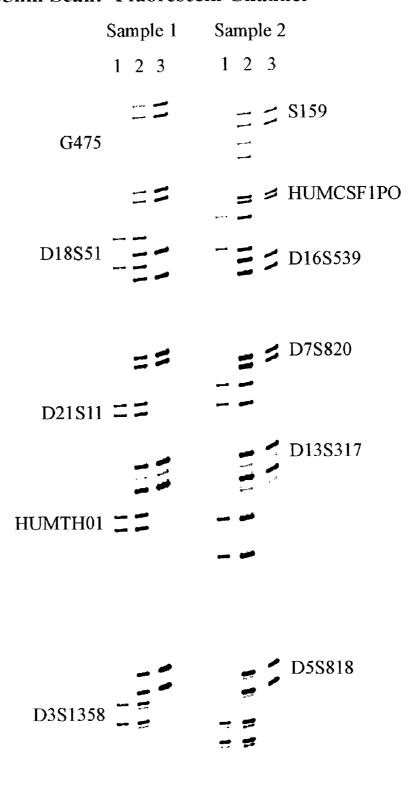
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FIG 4A

505nm Scan: Fluorescein Channel



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FIG 4B

585nm Scan: Tetramethyl Rhodamine Channel

Sample 1 Sample 2
1 2 3 1 2 3

HUMFIBRA --

HUMTPOX --

D8S1179

HUMvWFA31 ...

Amelogenin

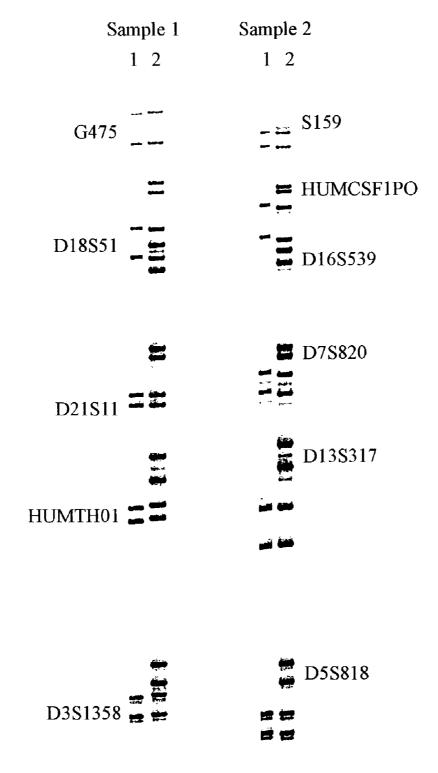
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FIG 5A

505nm Scan: Fluorescein Channel



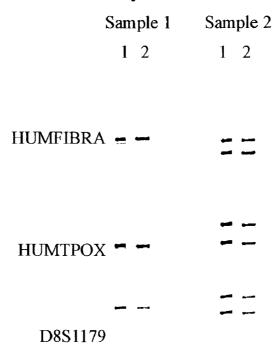
Mar. 7, 2006

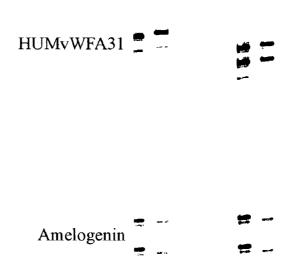
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FIG 5B

585nm Scan: Tetramethyl Rhodamine Channel





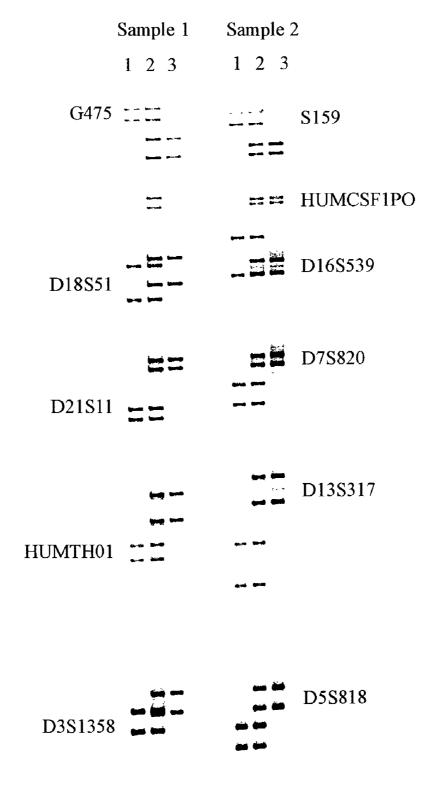
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FIG 6A

505nm Scan: Fluorescein Channel



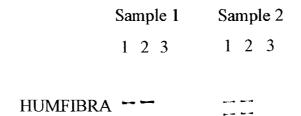
Mar. 7, 2006

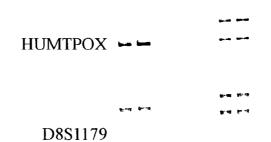
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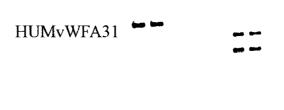
US 7,008,771 B1

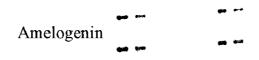
FIG 6B

585nm Scan: Tetramethyl Rhodamine Channel









1

MULTIPLEX AMPLIFICATION OF SHORT TANDEM REPEAT LOCI

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional of U.S. patent application Ser. No. 09/199,542, filed Nov. 25, 1998, now U.S. Pat. No. 6,479,235, issued Nov. 12, 2002, which is a continuationin-part of U.S. patent application Ser. No. 08/632,575, filed 10 Apr. 15, 1996, now U.S. Pat. No. 5,843,660, issued Dec. 1, 1998, which is a continuation-in-part of U.S. patent application Ser. No. 08/316,544, filed Sep. 30, 1994, now abandoned.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

FIELD OF THE INVENTION

The present invention is generally directed to the detection of genetic markers in a genomic system. The present invention is more specifically directed to the simultaneous 25 amplification of multiple distinct polymorphic genetic loci using the polymerase chain reaction or other amplification systems to determine, in one reaction, the alleles of each locus contained within the multiplex system.

BACKGROUND OF THE INVENTION

DNA typing is commonly used to identify the parentage of human children, and to confirm the lineage of horses, dogs, other animals, and agricultural crops. DNA typing is 35 also commonly employed to identify the source of blood, saliva, semen, and other tissue found at a crime scenes or other sites requiring identification of human remains. DNA typing is also employed in clinical settings to determine success or failure of bone marrow transplantation and pres- 40 ence of particular cancerous tissues. DNA typing involves the analysis of alleles of genomic DNA with characteristics of interest, commonly referred to as "markers". Most typing methods in use today are specifically designed to detect and more regions of DNA markers known to appear in at least two different forms in a population. Such length and/or sequence variation is referred to as "polymorphism." Any region (i.e. "locus") of DNA in which such a variation occurs is referred to as a "polymorphic locus." The methods 50 and materials of the present invention are designed for use in the detection of multiple loci of DNA, some or all of which are polymorphic loci.

Genetic markers which are sufficiently polymorphic with respect to length or sequence have long been sought for use 55 in identity applications, such as paternity testing and identification of tissue samples collected for forensic analysis. The discovery and development of such markers and methods for analyzing such markers have gone through several phases of development over the last several years.

The first identified DNA variant markers were simple base substitutions, i.e. simple sequence polymorphisms, which were most often detected by Southern hybridization assays. For examples of references describing the identification of such markers, designed to be used to analyze restriction 65 endonuclease-digested DNA with radioactive probes, see: Southern, E. M. (1975), J. Mol. Biol. 98(3):503-507;

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Schumm, et al. (1988), American Journal of Human Genetics 42:143-159; and Wyman, A. and White, R. (1980) Proc. Natl. Acad. Sci, U.S.A. 77:6754-6758.

The next generation of markers were size variants, i.e. length polymorphisms, specifically "variable number of tandem repeat" (VNTR) markers (Nakamura Y., et al. (1987), Science 235: 1616-1622; and U.S. Pat. No. 4,963, 663 issued to White et al. (1990); U.S. Pat. No. 5,411,859 continuation of 4,963,663 issued to White et al. (1995)) and "minisatellite" markers (Jeffreys et al. (1985a), Nature 314: 67-73; Jeffreys et al. (1985b) Nature 316:76-79, U.S. Pat. No. 5,175,082 for an invention by Jeffreys). Both VNTR and minisatellite markers, contain regions of nearly identical sequences repeated in tandem fashion. The core repeat sequence is 10 to 70 bases in length, with shorter core repeat sequences referred to as "minisatellite" repeats and longer repeats referred to as VNTRs. Different individuals in a human population contain different numbers of the repeats. The VNTR markers are generally more highly polymorphic than base substitution polymorphisms, sometimes displaying up to forty or more alleles at a single genetic locus. However, the tedious process of restriction enzyme digestion and subsequent Southern hybridization analysis are still required to detect and analyze most such markers.

The next advance involved the joining of the polymerase chain reaction (PCR) (U.S. Pat. No. 4,683,202 by Mullis, K. B.) technology with the analysis of VNTR loci (Kasai, K. et al. (1990) Journal Forensic Science 35(5):1196-1200). 30 Amplifiable VNTR loci were discovered, which could be detected without the need for Southern transfer. The amplified products are separated through agarose or polyacrylamide gels and detected by incorporation of radioactivity during the amplification or by post-staining with silver or ethidium bromide. However, PCR can only be used to amplify relatively small DNA segments reliably, i.e. only reliably amplifying DNA segments under 3,000 bases in length Ponce, M & Micol, L. (1992) NAR 20(3):623; Decorte R, et al. (1990) DNA Cell Biol. 9(6):461-469). Consequently, very few amplifiable VNTRs have been developed.

In recent years, the discovery and development of polymorphic short tandem repeats (STRs) as genetic markers has stimulated progress in the development of linkage maps, the identification and characterization of diseased genes, and the analyze differences in the length and/or sequence of one or 45 simplification and precision of DNA typing. Specifically, with the discovery and development of polymorphic markers containing dinucleotide repeats (Litt and Luty (1989) Am J. Hum Genet 3(4):599-605; Tautz, D (1989) NAR 17:6463-6471; Weber and May (1989) Am J Hum Genet 44:388-396; German Pat. No. DE 38 34 636 C2, inventor Tautz, D; U.S. Pat. No. 5,582,979 filed by Weber, L.), STRs with repeat units of three to four nucleotides (Edwards, A., et al. (1991) Am. J. Hum. Genet. 49: 746-756.; Hammond, H. A., et al. (1994) Am. J. Hum. Genet. 55: 175-189; Fregeau, C. J.; and Fourney, R. M. (1993) BioTechniques 15(1): 100-119.; Schumm, J. W. et al. (1994) in The Fourth International Symposium on Human Identification 1993, pp. 177-187 (pub. by Promega Corp., 1994); and U.S. Pat. No. 5,364,759 by Caskey et al.; German Pat. No. DE 38 34 636 C2 by Tautz, D.) and STRs with repeat units of five to seven bases (See, e.g. Edwards et al. (1991) Nucleic Acids Res. 19:4791; Chen et al. (1993) Genomics 15(3): 621-5; Harada et al. (1994) Am. J. Hum. Genet. 55: 175-189; Comings et al. (1995), Genomics 29(2):390-6; and Utah Marker Development Group (1995), Am. J. Genet. 57:619-628; and Jurka and Pethiyagoda (1995) J. Mol. Evol. 40:120-126)), many of the deficiencies of previous methods have been over-

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come. STR markers are generally shorter than VNTR markers, making them better substrates for amplification than most VNTR markers.

STR loci are similar to amplifiable VNTR loci in that the amplified alleles at each such locus may be differentiated 5 based on length variation. Generally speaking STR loci are less polymorphic at each individual locus than VNTR loci. Thus, it is desirable to amplify and detect multiple STR systems in a single amplification reaction and separation to provide information for several loci simultaneously. Sys- 10 tems containing several loci are called multiplex systems and many such systems containing up to 11 separate STR loci have been described. See, e.g., Proceedings: American Academy of Forensic Sciences (Feb. 9-14, 1998), Schumm, James W. et al., p. 53, B88; Id., Gibson, Sandra D. et al., p. 15 53, B89; Id., Lazaruk, Katherine et al., p. 51, B83; Sparkes, R. et al., Int J Legal Med (1996) 109:186-194; AmpFlSTR Profiler™ PCR Amplification Kit User's Manual (1997), pub by Perkin-Elmer Corp, i-viii and 1-1 to 1-10; Amp-FISTR Profiler Plus™ PCR Amplification Kit User's 20 Manual (1997), pub by Perkin-Elmer Corp., i viii and 1—1 to 1–10; AmpFlSTR COfiler™ PCR Amplification Kit User Bulletin (1998), pub by Perkin-Elmer Corp. i-iii and 1—1 to 1–10; 9th International Symposium on Human Identification (Oct. 7-10, 1998), pub. by Promega Corp., Staub, Rick W. 25 et al., Poster Abstract 15; Id., Willard, Jeanne M. et al., Poster Abstract 73; and Id., Walsh, P. Sean, et al., Speaker Abstract for 8:50 am-9:20 am, Thursday, Oct. 8, 1998.

Amplification protocols with STR loci can be designed to produce small products, generally from 60 to 500 base pairs 30 (bp) in length, and alleles from each locus are often contained within a range of less than 100 bp. This allows simultaneous electrophoretic analysis of several systems on the same gel or capillary electrophoresis by careful design of PCR primers such that all potential amplification products from an individual system do not overlap the range of alleles of other systems. Design of these systems is limited, in part, by the difficulty in separating multiple loci in a single gel or capillary. This occurs because there is spacial compression of fragments of different sizes, especially longer fragments 40 in gels or capillaries, i.e., commonly used means for separation of DNA fragments by those skilled in the art.

The United States Federal Bureau of Investigation ("FBI") has established and maintains a Combined DNA Index System ("CODIS"), a database of DNA typing infor- 45 mation. Local, state, and national law enforcement agencies use the CODIS system to match forensic DNA evidence collected at crime scenes with DNA information in the database. CODIS and other national database systems have proven to be an effective tool for such agencies to use in 50 solving violent crimes. (See, e.g. Niezgoda, Stephen, in Cambridge Healthtech Institute's Second Annual Conference on DNA Forensics: Science, Evidence, and Future Prospects (Nov. 17–18, 1998), pp. 1–21.; Niezgoda, Stephen in Proceedings From The Eighth International Symposium 55 on Human Identification 1997, pub. by Promega Corporation (1998), pp 48-49; Frazier, Rachel R. E. et al. Id., pp. 56-60; Niezgoda, S. J. Profiles in DNA 1(3): 12-13; Werrett, D. J. and Sparkes, R. in Speaker Abstracts: 9th International Symposium on Human Identification (Oct. 7-10, 1998) pp. 60 5-6). Until recently, only restriction fragment length polymorphism ("RFLP") data obtained from the analysis of particular VNTR loci was considered a core component in the database. The FBI has recently identified thirteen polymorphic STR loci for inclusion in the CODIS database. The 65 thirteen CODIS STR loci are HUMCSF1PO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51,

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D21S11, HUMFIBRA, HUMTH01, HUMTPOX, and HUMvWFA31. (Budowle, Bruce and Moretti, Tamyra in Speaker Abstracts: 9th International Symposium on Human Identification (Oct. 7–10, 1998) pp. 7–8). Both VNTR and STR marker data are currently maintained in the CODIS database. (See, e.g. Niezgoda, Stephen in Second Annual Conference on DNA Forensics, supra). Until the present invention, the number of loci which could be co-amplified in a single reaction, and analyzed thereafter was limited. Specifically, no materials or methods had been developed for use in multiplex amplification of thirteen or more STR loci, much less the thirteen polymorphic STR loci identified for use in the CODIS database.

The materials and methods of the present method are designed for use in multiplex analysis of particular polymorphic loci of DNA of various types, including single-stranded and double-stranded DNA from a variety of different sources. The present invention represents a significant improvement over existing technology, bringing increased power of discrimination, precision, and throughput to DNA profiling for linkage analysis, criminal justice, paternity testing, and other forensic, medical, and genetic identification applications.

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide a method and materials for the simultaneous amplification of sets of loci, which include multiple distinct polymorphic short tandem repeat (STR) loci, in a single multiplex reaction, using PCR or other amplification systems in combination with gel electrophoresis, capillary electrophoresis or other separation and detection methods to analyze and compare the relative lengths of the alleles of each locus amplified in the multiplex reaction. Multiplex analysis of the sets of loci disclosed herein has not been previously described in the prior art. There has also not been any previous description of the sequences for many of the primers disclosed herein below, all of which are shown to be useful in multiplex amplification of the sets of loci disclosed.

It is also an object of the present invention to provide a method, a kit, and primers specific for multiplex amplifications comprising specified loci.

These and other objects are addressed by the present invention which is directed to a method and materials for simultaneously analyzing or determining the alleles present at each individual locus of each multiplex. In general, the method of this invention comprises the steps of (a) obtaining at least one DNA sample to be analyzed, wherein the DNA sample has at least thirteen loci which can be co-amplified; (b) co-amplifying the at least thirteen loci of the DNA sample; and (c) detecting the amplified materials in a fashion which reveals the polymorphic nature of the systems employed.

In one embodiment, the present invention is a method of simultaneously determining the alleles present in a set of loci from one or more DNA samples, comprising the steps of:

- (a) obtaining at least one DNA sample to be analyzed;
- (b) selecting a set of loci of the DNA sample, comprising at least thirteen short tandem repeat loci which can be co-amplified;
- (c) co-amplifying the loci in the set in a multiplex amplification reaction, wherein the product of the reaction is a mixture of amplified alleles from each of the co-amplified loci in the set; and

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(d) evaluating the amplified alleles in the mixture to determine the alleles present at each of the loci analyzed in the set within the DNA sample.

At least four of the at least thirteen short tandem repeat loci are preferably selected from the group of loci consisting of: D3S1539, D4S2368, D5S818, D7S820, D9S930, D10S1239, D13S317, D14S118, D14S548, D14S562, D16S490, D16S539, D16S753, D17S1298, D17S1299, D19S253, D20S481, D22S683, HUMCSF1PO, HUMTPOX, HUMTH01, HUMF13AO1, HUMBFXIII, HUMLIPOL, HUMvWFA31.

In another embodiment of the invention, the set of loci selected in step (b) of In another embodiment of the invention, the set of loci selected in step (b) of the method 15 comprises thirteen CODIS STR loci (i.e., D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, and HUMvWFA31) which can be co-amplified and analyzed by themselves, or with additional loci using 20 methods of the present invention.

In a further aspect, this invention is a kit for simultaneously analyzing a set of loci of genomic DNA, comprising oligonucleotide primers for co-amplifying a set of loci of the genomic DNA to be analyzed, wherein the set of loci 25 comprises at least thirteen short tandem repeat loci which can be co-amplified in the same multiplex reaction, and wherein the primers are in one or more containers. More preferably, the kit comprises oligonucleotide primer pairs for co-amplifying a set of at least thirteen loci of human 30 genomic DNA, the set of loci comprising D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, and HUMvWFA31.

In yet a further aspect, the invention is primer sequences 35 and primer pairs for amplifying specific loci of human DNA. Use of the primers and primer pairs of this invention for multiplex analysis of human DNA is demonstrated herein, below. The primers of this invention are suitable for use in the method of this invention, wherein they can be used in 40 labeled form, as noted below, to assist the evaluation step of the method.

The approaches specified in the present invention produce savings of time, labor, and materials in the analysis of loci contained within the multiplexes. The method of the present 45 invention allows thirteen or more, even as many as sixteen or more, loci to be co-amplified in one tube using a single amplification reaction, instead of amplifying each locus independently in separate tubes or in smaller groups of loci.

The present invention has specific use in the field of 50 forensic analysis, paternity determination, monitoring of bone marrow transplantation, linkage mapping, and detection of genetic diseases and cancers. By allowing thirteen methods of the present invention significantly increase the certainty with which one can match DNA prepared from 55 different samples from the same individual. The need to match or distinguish accurately between samples containing very small amounts of DNA is particularly acute in forensics applications, where many convictions (and acquittals) turn on DNA typing analysis.

Scientists, particularly forensic scientists, have long appreciated the need to analyze multiple polymorphic loci of DNA in order to ensure that a match between two samples of DNA is statistically significant. (Presley, L. A. et al., in *The Third International Symposium on Human Identification* 65 1992, pp. 245–269 (pub. by Promega Corp., 1993); Bever, R. A., et al., in *The Second International Symposium on*

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Human Identification 1991, pp. 103–128. (pub. by Promega Corp., 1992)) However, until this invention, one could not simultaneously analyze thirteen or more STR loci in a single reaction. To realize the importance of such multiplexing capabilities, it helps to understand some of the mathematics behind DNA typing analysis.

For purposes of illustration, suppose every STR locus has a genotype (i.e., pattern of two alleles) frequency of one in ten. In other words, suppose that the chance of two randomly selected individuals have a matching type for a single STR is 1/10. However, if two different STR loci are analyzed, the chance of a random match with both systems is 1/100. If three STR loci are analyzed, the chances of a random match with each of the three systems is 1/1,000 and so on. Consequently, it is easy to see how increasing the number of STR loci analyzed reduces the likelihood of random matches within the general population, thereby increasing the chance one can accurately identify a suspect's presence at a crime scene by comparing the individual's type with crime scene evidence. Similar reasoning can be used to conclude that the method of this invention also would increase the likelihood of accurately identifying a suspected father in a paternity case, of correctly matching bone marrow tissue, of developing significant results from linkage mapping studies, and of detecting genetic diseases and cancers.

Further objects, features, and advantages of the invention will be apparent from the following best mode for carrying out the invention and the illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a plot of the output of three-color fluorescent detection of the products of simultaneous amplification of the loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, and HUMvWFA31 of a sample of human genomic DNA, as detected with the ABI PRISM® 310 Genetic Analyzer in Example 1.

FIG. 1B is a plot of the output of three-color fluorescent detection of a control sample processed the same way as FIG. 1A, with no genomic DNA in the amplification reaction

FIG. 2A is a plot of the output of three-color fluorescent detection of the products of simultaneous amplification of the loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, HUMvWFA31, G475, S159, and Amelogenin of a sample of human genomic DNA, as detected with the ABI PRISM 310 Genetic Analyzer in Example 2.

FIG. 2B is a plot of the output of three-color fluorescent detection of a control sample processed the same way as FIG. 2A, with no genomic DNA substrate in the amplification reaction.

FIG. 3A is a plot of the output of three-color fluorescent detection of the products of simultaneous amplification of the loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21 S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, HUMvWFA31, G475, S159, and Amelogenin of a sample of human genomic DNA, as detected with an ABI PRISM® 377 DNA Sequencer in Example 3.

FIG. 3B is a plot of the output of three-color fluorescent detection of a control sample processed the same way as FIG. 3A, with no genomic DNA substrate in the amplification reaction.

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FIGS. 4A and 4B are laser printed images of the results of fluorescent detection of the products of simultaneous amplification of the loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, HUMvWFA31, 5 G475, S159, and Amelogenin as detected using the fluorescein channel (FIG. 4A) and carboxy-tetramethylrhodamine channel (FIG. 4B) of a Hitachi FMBIO® II Fluorescent Scanner, as described in Example 4.

FIGS. 5A and 5B are laser printed images of the results of fluorescent detection of the products of simultaneous amplification of the loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, HUMvWFA31, G475, S159, and Amelogenin as detected using the fluorescein channel (FIG. 5A) and carboxy-tetramethylrhodamine channel (FIG. 5B) of a Hitachi FMBIO® II Fluorescent Scanner, as described in Example 5.

FIGS. 6A and 6B are laser printed images of the results of fluorescent detection of the products of simultaneous amplification of the loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, HUMvWFA31, C221, S159, and Amelogenin as detected using the fluorescein channel (FIG. 6A) and carboxy-tetramethylrhodamine 25 channel (FIG. 6B) of a Hitachi FMBIO® II Fluorescent Scanner, as described in Example 6.

DETAILED DESCRIPTION OF THE INVENTION

A. Definitions

The following definitions are intended to assist in providing a clear and consistent understanding of the scope and detail of the following terms, as used to describe and define the present invention:

"Allelic ladder": a standard size marker consisting of amplified alleles from the locus.

"Allele": a genetic variation associated with a segment of DNA, i.e., one of two or more alternate forms of a DNA sequence occupying the same locus.

"Biochemical nomenclature": standard biochemical nomenclature is used herein in which the nucleotide bases are designated as adenine (A); thymine (T); guanine (G); and cytosine (C). Corresponding nucleotides are, for example, deoxyguanosine-5'-triphosphate (dGTP).

"DNA polymorphism": the condition in which two or more different nucleotide sequences in a DNA sequence coexist in the same interbreeding population.

"Locus" or "genetic locus": a specific position on a chromosome. Alleles of a locus are located at identical sites on homologous chromosomes.

"Locus-specific primer": a primer that specifically hybridizes with a portion of the stated locus or its complementary 55 strand, at least for one allele of the locus, and does not hybridize efficiently with other DNA sequences under the conditions used in the amplification method.

"Pentanucleotide tandem repeat": a subclass of the STR polymorphisms defined below. Unless specified otherwise, 60 the term "pentanucleotide tandem repeat" encompasses perfect STRs wherein the repeat unit is a five base sequence, and imperfect STRs wherein at least one repeat unit is a five base repeat.

"Polymerase chain reaction" or "PCR": a technique in 65 which cycles of denaturation, annealing with primer, and extension with DNA polymerase are used to amplify the

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number of copies of a target DNA sequence by approximately 10⁶ times or more. The polymerase chain reaction process for amplifying nucleic acid is covered by U.S. Pat. Nos. 4,683,195 and 4,683,202, which are incorporated herein by reference for a description of the process.

"Polymorphic short tandem repeat loci": STR loci, defined below, in which the number of repetitive sequence elements (and net length of sequence) in a particular region of genomic DNA varies from allele to allele, and from individual to individual.

"Polymorphism information content" or "PIC": a measure of the amount of polymorphism present at a locus (Botstein et al., 1980). PIC values range from 0 to 1.0, with higher values indicating greater degrees of polymorphism. This measure generally displays smaller values than the other commonly used measure, i.e., heterozygosity. For markers that are highly informative (heterozygosities exceeding about 70%), the difference between heterozygosity and PIC is slight.

"Primer": a single-stranded oligonucleotide or DNA fragment which hybridizes with a DNA strand of a locus in such a manner that the 3' terminus of the primer may act as a site of polymerization using a DNA polymerase enzyme.

"Primer pair": two primers including, primer 1 that hybridizes to a single strand at one end of the DNA sequence to be amplified and primer 2 that hybridizes with the other end on the complementary strand of the DNA sequence to be amplified.

"Primer site": the area of the target DNA to which a primer hybridizes.

"Short tandem repeat loci" or "STR loci": regions of genomic DNA which contain short, repetitive sequence elements of 3 to 7 base pairs in length. The term STR also encompasses a region of genomic DNA wherein more than a single three to seven base sequence is repeated in tandem or with intervening bases, provided that at least one of the sequences is repeated at least two times in tandem. Each sequence repeated at least once within an STR is referred to herein as a "repeat unit."

The sequences of the STR loci analyzed using the materials and methods of the present invention can be divided into two general categories, perfect and imperfect. The term "perfect" STR, as used herein, refers to a region of doublestranded DNA containing a single three to seven base repeat unit repeated in tandem at least two times, e.g. (AAAAT)₂. The term "imperfect" STR, as used herein, refers to a region of DNA containing at least two tandem repeats of a perfect repeat unit and at least one repeat of an imperfect repeat unit, wherein the imperfect repeat unit consists of a DNA sequence which could result from one, two, three, or four base insertions, deletions, or substitutions in the sequence of the perfect repeat unit, e.g. (AAAAT)₁₂(AAAAAT)₅AAT (AAATT)₄. Every imperfect STR sequence contains at least one perfect STR sequence. Specifically, every STR sequence, whether perfect or imperfect, includes at least one repeat unit sequence appearing at least two times in tandem, a repeat unit sequence which can be represented by formula (I):

$$(A_2G_xT_vC_z)_n$$
 (I)

wherein A, G, T, and C represent the nucleotides which can be in any order; w, x, y and z represent the number of each nucleotide in the sequence and range from 0 to 7 with the sum of w+x+y+z ranging between 3 and 7; and n represents the number of times the sequence is tandemly repeated and is at least 2.

B. Selection of Multiplex Reaction Components

The method of the present invention contemplates selecting an appropriate set of loci, primers, and amplification protocols to generate amplified alleles from multiple coamplified loci which preferably do not overlap in size or, 5 more preferably, which are labeled in a way which enables one to differentiate between the alleles from different loci which overlap in size. In addition, this method contemplates the selection of short tandem repeat loci which are compatible for use with a single amplification protocol. The specific 10 combinations of loci described herein are unique in this application. Combinations of loci may be rejected for either of the above two reasons, or because, in combination, one or more of the loci do not produce adequate product yield, or fragments which do not represent authentic alleles are 15 produced in this reaction.

Successful combinations in addition to those disclosed herein can be generated by trial and error of locus combinations, by selection of primer pair sequences, and by adjustment of primer concentrations to identify an equilib- 20 rium in which all included loci may be amplified. Once the method and materials of this invention are disclosed, various methods of selecting loci, primer pairs, and amplification techniques for use in the method and kit of this invention are likely to be suggested to one skilled in the art. All such 25 methods are intended to be within the scope of the appended

Of particular importance in the practice of the method of this invention is the size range of amplified alleles produced from the individual loci which are co-amplified in the 30 multiplex amplification reaction step. For ease of analysis with current technologies, systems which can be detected by amplification of fragments smaller than 500 bases are most preferable.

Practice of the method of the present invention begins 35 with selection of a set of loci comprising at least thirteen STR loci, which can be co-amplified in a single multiplex amplification reaction. Selection of loci and oligonucleotide primers used to amplify the loci in the multiplex amplification reaction of the present method is described herein 40 below, and illustrated in the Examples below.

C. Use of Multiplexes of Three Loci to Develop Multiplexes Using More than Three Loci

to select a set of loci for use in the present invention. One preferred technique for developing useful sets of loci for use in this method of analysis is described below. Once a multiplex containing three STR loci is developed, it may be used as a core to create multiplexes containing more than 50 three loci. New combinations of more than three loci can, thus, be created which include the first three loci. For example, the core multiplex containing loci D7S820, D13S317, and D5S818 was used to generate derivative multiplexes of:

D16S539, D7S820, D13S317, and D5S818;

HUMCSF1PO, HUMTPOX, D16S539, D7S820, D13S317, and D5S818;

HUMCSF1PO, HUMTPOX, HUMTH01, D16S539, D7S820, D13S317, and D5S818;

HUMCSF1PO, HUMTPOX, HUMTH01, HUMvWFA31, D16S539, D7S820, D13S317, and D5S818; D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFI-BRA, HUMTH01, HUMTPOX, and HUMvWA31; S159, HUMCSF1P0, D16S539, D7S820, D13S317, and

D5S818; D3S1358, D5S818, D7S820, D8S1179,

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D16S539, D13S317, D18S51, D21S11. HUMCSF1PO, HUMFIBRA, HUMTH01, HUMT-POX, and HUMvWFA31; and

D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFI-BRA, HUMTH01, HUMTPOX, HUMvWFA31, G475, S159, and Amelogenin.

It is contemplated that core sets of loci can be used to generate other appropriate derivative sets of STR loci for multiplex analysis using the method of this invention. Regardless of what method is used to select the loci analyzed using the method of the present invention, all the loci selected for multiplex analysis share the following characteristics: (1) they produce sufficient amplification product to allow evaluation; (2) they generate few if any artifacts due to the addition (or lack of addition) of a base to the amplified alleles during the multiplex amplification step; (3) they generate few, if any, artifacts due to premature termination of amplification reactions by a polymerase; and (4) they produce little or no "trailing" bands of smaller molecular weight from consecutive single base deletions below a given authentic amplified allele. See, e.g., Schumm et al., Fourth International Symposium on Human Identification 1993, pp. 177-187 (pub. by Promega Corp., 1994).

The same technique used to identify the set of at least three loci, described above, can be applied to select thirteen or more loci of human genomic DNA or multiplex analysis, according to a preferred embodiment of the method of analysis of the present invention. Any set of loci identified as described above is suitable for multiplex analysis in accordance with the present invention, provided the set of loci comprises at least thirteen STR loci. More preferably, at least four of the at least thirteen STR loci analyzed according to the present invention are selected from the group of loci consisting of:

D3S1539, D4S2368, D5S818, D7S820, D9S930, D10S1239, D13S317, D14S118, D14S548, D14S562, D16S490, D16S539, D16S753, D17S1298, D17S1299, D19S253, D20S481, D22S683, HUMCSF1PO, HUMTPOX. HUMTH01, HUMF13AO1. HUMBFXIII, HUMLIPOL, and HUMvWFA31.

Even more preferably, the set of loci analyzed according to the present invention includes all thirteen CODIS loci, i.e. D3S1358, D5S818, D7S820, D8S1179, D13S317, Any one of a number of different techniques can be used 45 D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, and HUMvWFA31.

> At least one of the loci selected for co-amplification in the present multiplex reaction is preferably an STR locus with a repeat unit of five to seven bases or base pairs in length, more preferably an STR locus with a pentanucleotide repeat. As is demonstrated in U.S. patent application Ser. No. 09/018,584, which is incorporated by reference herein, loci with such intermediate length repeats can be amplified with minimal incidence of artifacts, e.g. due to repeat slippage. Three such loci with pentanucleotide repeats, G475, C221 and S159, are included in the sets of loci identified immediately above. The terms "G475", "C221", and "S159", as used herein, refer to names assigned to pentanucleotide repeat loci identified, as described in U.S. patent application Ser. No. 09/018,584, incorporated by reference above. Each name corresponds to a clone from which each pentanucleotide locus was identified. The sequence of the G475 clone, described therein as SEQ ID NO:34, is identified herein as SEQ ID NO:108. The sequence of the C221 clone, described 65 therein as SEQ ID NO:2, is identified herein as SEQ ID NO:109. The sequence of the S159 clone, described therein as SEQ ID NO: 26, is identified herein as SEQ ID NO:110.

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Individual primers and primer pairs identified for use in amplifying G475, C221, and S159 therein can also be used to amplify the same loci in the sets of at least thirteen loci co-amplified and analyzed according to the present invention.

The set of loci selected for co-amplification and analysis according to the invention preferably further comprises at least one locus in addition to the at least thirteen STR loci. The additional locus preferably includes a sequence polymorphism, or another feature which identifies a particular 10 characteristic which separates the DNA of an individual from the DNA of other individuals in the population. The additional locus more preferably is a locus which identifies the gender of the source of the DNA sample analyzed. When the DNA sample is human genomic DNA, a gender identi- 15 fying locus such as the Amelogenin locus is preferably selected for co-amplification and analysis according to the present method. The Amelogenin locus is identified by GenBank as HUMAMELY (when used to identify a locus on the Y chromosome contained in male DNA) or as HUMA- 20 MELX (when used to identify a locus on the X chromosome in male or female DNA). When the Amelogenin locus is co-amplified in the same multiplex amplification reaction as the set of at least thirteen short tandem repeat loci, the sequence of at least one of the primers used to amplify this 25 particular locus in the multiplex amplification reaction preferably has a sequence selected from: SEQ ID NO:86, SEQ ID NO:105, and SEQ ID NO:87.

D. Selection of Primers

Once a set of loci for co-amplification in a single multiplex reaction is identified, one can determine primers suitable for co-amplifying each locus in the set. Care should be used in selecting the sequence of primers used in the multiplex reaction. Inappropriate selection of primers can produce several undesirable effects such as lack of amplification, amplification at multiple sites, primer dimer formation, undesirable interaction of primer sequences from different loci, production of alleles from one locus which overlap with alleles from another, or the need for amplification conditions or protocols for the different loci which are incompatible in a multiplex. Primers used in the present method or included in the present kits of the invention are preferably selected according to the following selection process.

Primers are preferably developed and selected for use in the multiplex systems of the invention by employing a re-iterative process of selecting primer sequences, mixing the primers for co-amplification of the selected loci, co-amplifying the loci, then separating and detecting the amplified products. Initially, this process often produces the amplified alleles in an imbalanced fashion (i.e., higher product yield for some loci than for others) and may also generate amplification products which do not represent the alleles themselves. These extra fragments may result from 55 any number of causes described above.

To eliminate such extra fragments from the multiplex systems, individual primers from the total set are used with primers from the same or other loci to identify which primers contribute to the amplification of the extra fragments. Once two primers which generate one or more of the fragments are identified, one or both contributors are modified and retested, either in a pair alone or in the multiplex system (or a subset of the multiplex system). This process is repeated until evaluation of the products yields amplified 65 alleles with no or an acceptable level of extra fragments in the multiplex system.

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On occasion, extra fragments can be eliminated by labeling the opposite primer in a primer pair. This change reveals the products of the opposing primer in the detection step. This newly labeled primer may amplify the true alleles with greater fidelity than the previously labeled primer generating the true alleles as a greater proportion of the total amplification product.

The determination of primer concentration may be performed either before or after selection of the final primer sequences, but is preferably performed after that selection. Generally, increasing primer concentration for any particular locus increases the amount of product generated for that locus. However, this is also a re-iterative process because increasing yield for one locus may decrease it for one or more other loci. Furthermore, primers may interact directly affecting yield of the other loci. Linear increases in primer concentration do not necessarily produce linear increases in product yield for the corresponding locus.

Locus to locus balance is also affected by a number of parameters of the amplification protocol such as the amount of template used, the number of cycles of amplification, the annealing temperature of the thermal cycling protocol, and the inclusion or exclusion of an extra extension step at the end of the cycling process. Absolutely even balance across all alleles and loci is generally not achieved.

The process of multiplex system development may also be a re-iterative process in another sense described, above. That is, it is possible, first, to develop a multiplex system for a small number of loci, this system being free or nearly free of extra fragments from amplification. Primers of this system may be combined with primers for one or more additional loci. This expanded primer combination may or may not produce extra fragments from amplification. In turn, new primers may be introduced and evaluated.

One or more of the re-iterative selection processes described above are repeated until a complete set of primers is identified which can be used to co-amplify the at least thirteen loci selected for co-amplification as described above. It is understood that many different sets of primers may be developed to amplify a particular set of loci.

Synthesis of the primers used in the present method can be conducted using any standard procedure for oligonucleotide synthesis known to those skilled in the art. At least one primer for each locus is preferably covalently attached to a dye label, as described in Section F, below.

Table 1, below, provides a list sequences of primers which have been determined to be suitable for use in amplifying the corresponding polymorphic STR loci listed therein. At least one primer listed in Table 1 is preferably used to amplify at least one of the loci selected for co-amplification and analysis as described above. It is understood that other primers could be identified which are suitable for simultaneous amplification of the loci listed below.

TABLE 1

Locus	Primer SEQ ID NO:'s
D7S820	1, 2, 80 and 81
D13S317	3, 4, 82 and 83
D5S818	5, 6, 84 and 85
D3S1539	7, 8 and 49
D17S1298	9 and 10
D20S481	11, 12, 52 and 53
D9S930	13, 14, 55 and 61
D10S1239	15, 16 and 54
D14S118	17 and 18
D14S562	19 and 20

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TABLE 1-continued

Locus	Primer SEQ ID NO:'s	
D14S548	21 and 22	
D16S490	23 and 24	
D16S753	25 and 26	
D17S1299	27 and 28	
D16S539	29, 30, 58, 79 and 97	
D22S683	31 and 32	
HUMCSF1PO	33, 34, 77, 78 and 98	
HUMTPOX	35, 36, 72 and 73	
HUMTH01	37, 38, 66, 67 and 103	
HUMvWFA31	39, 40, 59, 60 and 76	
HUMF13A01	41 and 42	
HUMFESFPS	43 and 44	
HUMBFXIII	45 and 46	
HUMLIPOL	47 and 48	
D19S253	50 and 51	
D4S2368	56 and 57	
D18S51	62, 63, 101 and 102	
D21S11	64 and 65	
D3S1358	68, 69 and 106	
HUMFIBRA	70, 71 and 107	
D8S1179	74, 75 and 104	
G475	88, 89 and 94	
S159	90, 91, 92, 93, 95 and 96	
C221	99 and 100	

E. Preparation of DNA Samples

Samples of genomic DNA can be prepared for use in the method of this invention using any method of DNA preparation which is compatible with the amplification of DNA. 30 Many such methods are known by those skilled in the art. Examples include, but are not limited to DNA purification by phenol extraction (Sambrook, J., et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp. 9.14-9.19), and partial purification by salt precipitation (Miller, S. et al. (1988) Nucl. Acids Res. 16:1215) or chelex (Walsh et al., (1991) BioTechniques 10:506-513, Comey, et al., (1994) Forensic Sci. 39:1254) and the release of unpurified material using untreated blood (Burckhardt, J. (1994) PCR Methods and Applications 3:239-243, McCabe, Edward R. B.,(1991) PCR Methods and Applications 1:99-106, Nordvåg, Bjørn-Yngvar (1992) BioTechniques 12:4 pp. 490-492).

When the at least one DNA sample to be analyzed using the method of this invention is human genomic DNA, the DNA is preferably prepared from tissue, selected from the group consisting of blood, semen, vaginal cells, hair, saliva, urine, bone, buccal samples, amniotic fluid containing placental cells or fetal cells, chorionic villus, and mixtures of any of the tissues listed above.

Optionally, DNA concentrations can be measured prior to use in the method of the present invention, using any standard method of DNA quantification known to those 55 skilled in the art. In such cases, the DNA concentration is preferably determined by spectrophotometric measurement as described by Sambrook, J., et al. (1989), supra, Appendix E.5, or fluorometrically using a measurement technique such as that described by Brunk C. F., et al. (1979), *Anal Biochem* 60 92: 497–500. The DNA concentration is more preferably measured by comparison of the amount of hybridization of DNA standards with a human-specific probe such as that described by Waye, J. S., et al. (1991) "Sensitive and specific quantification of human genomic deoxyribonucleic acid 65 (DNA) in forensic science specimens: casework examples," *J. Forensic Sci.*, 36:1198–1203. Use of too much template

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DNA in the amplification reactions can produce artifacts which appear as extra bands which do not represent true alleles.

F. Amplification of DNA

Once a sample of genomic DNA is prepared, the targeted loci can be co-amplified in the multiplex amplification step of the present method. Any one of a number of different amplification methods can be used to amplify the loci, including, but not limited to, polymerase chain reaction (PCR) (Saiki, R. K., et al. (1985), Science 230: 1350-1354), transcription based amplification (Kwoh, D. Y., and Kwoh, T. J. (1990), American Biotechnology Laboratory, October, 1990) and strand displacement amplification (SDA) (Walker, G. T., et al. (1992) Proc. Natl. Acad. Sci., U.S.A. 89: 392-396). Preferably, the DNA sample is subjected to PCR amplification using primer pairs specific to each locus in the set. Reference is made to the Sequence Listing at the end of this specification for details of the primer sequences used in the Examples below, some of which sequences are alternative embodiments of this invention.

At least one primer for each locus is preferably covalently attached to a dye label, more preferably a fluorescent dye label. The primers and dyes attached thereto are preferably selected for the multiplex amplification reaction, such that alleles amplified using primers for each locus labeled with one color do not overlap the alleles of the other loci in the set co-amplified therein using primers labeled with the same color, when the alleles are separated, preferably, by gel or capillary electrophoresis.

In a particularly preferred embodiment of the method of the present invention, at least one primer for each locus co-amplified in the multiplex reaction is labeled with a fluorescent label prior to use in the reaction. Fluorescent labels suitable for attachment to primers for use in the present invention are commercially available. See, e.g. fluorescein and carboxy-tetramethylrhodamine labels and their chemical derivatives from PE Biosystems and Molecular Probes. Most preferably, at least three different labels are used to label the different primers used in the multiplex amplification reaction. When a size marker is included to evaluate the multiplex reaction, the primers used to prepare the size marker are preferably labeled with a different label from the primers used to amplify the loci of interest in the reaction.

Details of the most preferred amplification protocol for each of the most preferred combinations of loci for use in the method of this invention are given in the Examples below. Reference is also made to the Examples for additional details of the specific procedure relating to each multiplex. The sequences of the locus-specific primers used in the Examples include a number of nucleotides which, under the conditions used in the hybridization, are sufficient to hybridize with an allele of the locus to be amplified and to be essentially free from amplification of alleles of other loci. Reference is made to U.S. Pat. No. 5,192,659 to Simons, the teaching of which is incorporated herein by reference for a more detailed description of locus-specific primers.

G. Separation and Detection of DNA Fragments

Once a set of amplified alleles is produced from the multiplex amplification step of the present method, the amplified alleles are evaluated. The evaluation step of this method can be accomplished by any one of a number of different means, the most preferred of which are described below.

Electrophoresis is preferably used to separate the products of the multiplex amplification reaction, more preferably 15

capillary electrophoresis (see, e.g., Buel, Eric et al. (1998), *Journal of Forensic Sciences*; 43:(1) pp. 164–170) or denaturing polyacrylamide gel electrophoresis (see, e.g., Sambrook, J. et al. (1989) In *Molecular Cloning—A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory Press, pp. 13.45–1 3.57). Gel preparation and electrophoresis procedures and conditions for suitable for use in the evaluating step of the method of this invention are illustrated in the Examples, below. Separation of DNA fragments in a denaturing polyacrylamide gel and in capillary electrophoresis occurs based primarily on fragment size.

Once the amplified alleles are separated, the alleles and any other DNA in the gel or capillary (e.g., DNA size markers or an allelic ladder) can then be visualized and analyzed. Visualization of the DNA in the gel can be accomplished using any one of a number of prior art techniques, including silver staining or reporters such as radioisotopes, fluorescers, chemiluminescers and enzymes in combination with detectable substrates. However, the 20 preferred method for detection of multiplexes containing thirteen or more loci is fluorescence (see, e.g., Schumm, J. W. et al. in Proceedings from the Eighth International Symposium on Human Identification, (pub. 1998 by Promega Corporation), pp. 78-84; Buel, Eric et al. (1998), 25 supra.), wherein primers for each locus in the multiplexing reaction is followed by detection of the labeled products employing a fluorometric detector. The references cited above, which describe prior art methods of visualizing alleles, are incorporated by reference herein.

The alleles present in the DNA sample are preferably determined by comparison to a size standard such as a DNA marker or a locus-specific allelic ladder to determine the alleles present at each locus within the sample. The most preferred size marker for evaluation of a multiplex amplification containing two or more polymorphic STR loci consists of a combination of allelic ladders for each of the loci being evaluated. See, e.g., Puers, Christoph et al., (1993) Am J. Hum Genet. 53:953–958, Puers, Christoph, et al. (1994) Genomics 23:260–264. See also, U.S. Pat. No's 5,599,666; 5,674,686; and 5,783,406 for descriptions of allelic ladders suitable for use in the detection of STR loci, and methods of ladder construction disclosed therein.

Following the construction of allelic ladders for individual loci, these may be mixed and loaded for gel electrophoresis at the same time as the loading of amplified samples occurs. Each allelic ladder co-migrates with alleles in the sample from the corresponding locus.

The products of the multiplex reactions of the present invention can be evaluated using an internal lane standard, a specialized type of size marker configured to run in the same lane of a polyacrylamide gel or same capillary. The internal lane standard preferably consists of a series of fragments of known length. The internal lane standard more preferably is labeled with a fluorescent dye which is distinguishable from other dyes in the amplification reaction.

Following construction of the internal lane standard, this standard can also be mixed with amplified sample or allelic ladders and loaded for electrophoresis for comparison of 60 migration in different lanes of gel electrophoresis or different capillaries of capillary electrophoresis. Variation in the migration of the internal lane standard indicates variation in the performance of the separation medium. Quantitation of this difference and correlation with the allelic ladders allows 65 correction in the size determination of alleles in unknown samples.

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H. Preferred Detection Technique: Fluorescent Detection

In one of the most preferred embodiments of the method of this invention, fluorescent detection is used to evaluate the amplified alleles in the mixture produced by the multiplex amplification reaction. Below is a brief summary of how that method of detection preferably is practiced.

With the advent of automated fluorescent imaging, faster detection and analysis of multiplex amplification products can be achieved. For fluorescent analysis, one fluorescent labeled primer can be included in the amplification of each locus. Fluorescent labeled primers preferably suited for use in the present invention include the fluorescein-labeled (FL-), carboxy-tetramethylrhodamine-labeled (TMR-), and 5,6-carboxyrhodamine 6G-labeled (R6G) primers, such as are illustrated in the Examples, below. Separation of the amplified fragments produced using such labeled primers is achieved preferably by slab gel electrophoresis or capillary electrophoresis. The resulting separated fragments can be analyzed using fluorescence detection equipment such as an ABI PRISM® 310 Genetic Analyzer, an ABI PRISM® 377 DNA Sequencer (Applied Biosystems Division, Perkin Elmer, Foster City, Calif.), or a Hitachi FMBIO® II Fluorescent Scanner (Hitachi Software Engineering America, Ltd. South San Francisco, Calif.).

In summary, the method of this invention is most preferably practiced using fluorescent detection as the detection step. In this preferred method of detection, one or both of each pair of primers used in the multiplex amplification reaction has a fluorescent label attached thereto, and as a result, the amplified alleles produced from the amplification reaction are fluorescently labeled. In this most preferred embodiment of the invention, the amplified alleles are subsequently separated by capillary electrophoresis and the separated alleles visualized and analyzed using a fluorescent image analyzer.

Fluorescent detection is preferred over radioactive methods of labeling and detection, because it does not require the use of radioactive materials, and all the regulatory and safety problems which accompany the use of such materials.

Fluorescent detection employing labeled primers is also preferred over other non-radioactive methods of detection, such as silver staining, because fluorescent methods of detection generally reveal fewer amplification artifacts than silver staining. The smaller number of artifacts are due, in part, to the fact that only amplified strands of DNA with labels attached are detected in fluorescent detection, while both strands of every amplified allele of DNA produced from the multiplex amplification reaction is stained and detected using the silver staining method of detection.

I. Kit

The present invention is also directed to kits that utilize the process described above. A basic kit comprises a container having one or more locus-specific primers. Instructions for use optionally may be included.

Other optional kit components may include an allelic ladder directed to each of the specified loci, a sufficient quantity of enzyme for amplification, amplification buffer to facilitate the amplification, loading solution for preparation of the amplified material for electrophoresis, genomic DNA as a template control, a size marker to insure that materials migrate as anticipated in the separation medium, and a protocol and manual to educate the user and to limit error in use. The amounts of the various reagents in the kits also can be varied depending upon a number of factors, such as the optimum sensitivity of the process. It is within the scope of

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this invention to provide test kits for use in manual applications or test kits for use with automated detectors or analyzers.

EXAMPLES

The following Examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. The Examples are intended to be illustrative, and are not intended in any 10 way to otherwise limit the scope of the claims or protection granted by the patent.

The human genomic DNA samples assayed in the Example below were prepared from blood or tissue culture cells, using a standard procedure described by Miller and 15 Dykes in (Miller, S. et al. (1988) *Nucl. Acids Res.* 16:1215). The isolation and quantification methods described therein are generally known to those skilled in the art and are preferred, but not required, for application of the invention.

Each Example below is an example of the use of the 20 method of this invention, to determine simultaneously the alleles present in at least thirteen loci from one or more DNA samples of human genomic DNA. Each set of loci coamplified below includes the thirteen short tandem repeat loci identified for use in the CODIS system (i.e., D3S1358, 25 HUMTHO1, D21S11, D18S51, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D13S317, D7S820, D16S539, and HUMCSF1PO). Some sets of loci co-amplified below also include one or more additional short tandem repeat loci, such as loci with pentanucleotide repeats (e.g., 30 G475, S159, or C221), and a non-STR locus, Amelogenin.

Table 2 summarizes which set of loci was co-amplified in the multiplex amplification reaction described in each Example below. The table also indicates which primer pair was used to amplify each such locus in each such multiplex 35 reaction. One primer of each primer pair listed on Table 2 was fluorescently labeled prior to being used in the multiplex amplification reaction. In some cases, a different label was used to label primers to different loci, such that the alleles produced using the different primers could be distinguished from one another when detected with a laseractivated fluorescence detection device.

Three different fluorescent labels were used in the Examples below, described as "FL" to indicate fluorescein-labeled, "TMR" to indicate carboxy-tetramethylrhodamine-labeled, and "R6G" to indicate 5,6-carboxyrhodamine 6G in Table 2, below. Table 2 also indicates which primer of each pair of primers used in the multiplex amplification reaction was so labeled in each Example (e.g., "FL-69" means the primer with SEQ ID NO:69 was labeled at its 5' end with 50 fluorescein prior to being used in the multiplex amplification reaction). In the text of each of the Examples, however, the label abbreviation is placed immediately before the SEQ ID NO of the labeled primer used in the amplification reaction described therein (e.g., "FL-SEQ ID NO:2" instead of 55 "FL-2").

TABLE 2

Example	Loci Amplified	Primer Pair: SEQ ID NO's Used	Fluorescent Label(s) Used	60
1	D3S1358	68, 69	FL-69	_
	HUMTHO1 D21S11	66, 67 64, 65	FL-66 FL-65	
	D18S51	62, 63	FL-62	65
	HUMvWFA31 D8S1179	76, 40 74, 75	TMR-40 TMR-75	00

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TABLE 2-continued

Rample	
HUMFIBRA 70, 71 TMR-70 D55818 84, 85 R6G-85 D13S317 82, 83 R6G-83 D75820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 2, 3 D3S1358 68, 69 FL-69 HUMTHO1 66, 67 FL-66 D21S11 64, 65 FL-65 D18S51 62, 63 FL-62 G475 88, 89 FL-88 Amelogenin 86, 87 TMR-86 HUMVWFA31 76, 40 TMR-40 D8S1179 74, 75 TMR-75 HUMTPOX 72, 73 TMR-73 HUMFIBRA 70, 71 TMR-70 D5S818 84, 85 R6G-85 D13S317 82, 83 R6G-83 D7S820 80, 81 R6G-83 D7S820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-79 S159 90, 91 R6G-79	
D5S818 84, 85 R6G-85 D13S317 82, 83 R6G-83 D7S820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 2, 3 D3S1358 68, 69 FL-69 HUMTHO1 66, 67 FL-65 D18S51 62, 63 FL-62 G475 88, 89 FL-88 Amelogenin 86, 87 TMR-86 HUMvWFA31 76, 40 TMR-40 D8S1179 74, 75 TMR-73 HUMTPOX 72, 73 TMR-73 HUMFIBRA 70, 71 TMR-73 D5S818 84, 85 R6G-85 D13S317 82, 83 R6G-83 D7S820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
D13S317 82, 83 R6G-83 D75820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 2, 3 D3S1358 68, 69 FL-69 HUMTHO1 66, 67 FL-65 D21S11 64, 65 FL-65 D18S51 62, 63 FL-62 G475 88, 89 FL-88 Amelogenin 86, 87 TMR-86 HUMvWFA31 76, 40 TMR-40 D8S1179 74, 75 TMR-73 HUMTPOX 72, 73 TMR-73 HUMFIBRA 70, 71 TMR-70 D5S818 84, 85 R6G-85 D13S317 82, 83 R6G-83 D78820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
D78820 80, 81 R6G-80 D165539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 2, 3 D3S1358 68, 69 FL-69 HUMTHO1 66, 67 FL-66 D21S11 64, 65 FL-65 D18S51 62, 63 FL-62 G475 88, 89 FL-88 Amelogenin 86, 87 TMR-86 HUMvWFA31 76, 40 TMR-40 D8S1179 74, 75 TMR-73 HUMTPOX 72, 73 TMR-73 HUMFIBRA 70, 71 TMR-70 D5SS18 84, 85 R6G-85 D13S317 82, 83 R6G-83 D78820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 2, 3 D3S1358 68, 69 FL-69 HUMTHO1 66, 67 FL-66 D21S11 64, 65 FL-65 D18S51 62, 63 FL-62 G475 88, 89 FL-88 Amelogenin 86, 87 TMR-86 HUMVWFA31 76, 40 TMR-40 D8S1179 74, 75 TMR-75 HUMTPOX 72, 73 TMR-73 HUMFIBRA 70, 71 TMR-70 D5SS18 84, 85 R6G-85 D13S317 82, 83 R6G-83 D7S820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-79 S159 90, 91 R6G-91	
HUMCSFIPO 77, 78 R6G-78 2, 3 D3S1358 68, 69 FL-69 HUMTHO1 66, 67 FL-66 D21S11 64, 65 FL-65 D18S51 62, 63 FL-62 G475 88, 89 FL-88 Amelogenin 86, 87 TMR-86 HUMVWFA31 76, 40 TMR-40 D8S1179 74, 75 TMR-75 HUMTPOX 72, 73 TMR-73 HUMFIBRA 70, 71 TMR-70 D5S818 84, 85 R6G-85 D13S317 82, 83 R6G-83 D7S820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSFIPO 77, 78 R6G-79 S159 90, 91 R6G-91	
HUMTHO1 66, 67 FL-66 D21S11 64, 65 FL-65 D18S51 62, 63 FL-62 G475 88, 89 FL-88 Amelogenin 86, 87 TMR-86 HUMVWFA31 76, 40 TMR-40 D8S1179 74, 75 TMR-75 HUMTPOX 72, 73 TMR-73 HUMFIBRA 70, 71 TMR-70 D5SS18 84, 85 R6G-85 D13S317 82, 83 R6G-83 D75820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-79 S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
D21S11 64, 65 FL-65 D18S51 62, 63 FL-62 G475 88, 89 FL-88 Amelogenin 86, 87 TMR-86 HUMvWFA31 76, 40 TMR-40 D8S1179 74, 75 TMR-73 HUMTPOX 72, 73 TMR-73 HUMFIBRA 70, 71 TMR-70 D5S818 84, 85 R6G-85 D13S317 82, 83 R6G-83 D78820 80, 81 R6G-80 D165539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-79 S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
D18S51 62, 63 FL-62 G475 88, 89 FL-88 Amelogenin 86, 87 TMR-86 HUMvWFA31 76, 40 TMR-70 D8S1179 74, 75 TMR-73 HUMTPOX 72, 73 TMR-73 HUMFIBRA 70, 71 TMR-70 D5S818 84, 85 R6G-85 D13S317 82, 83 R6G-83 D7S820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
G475 88, 89 FL-88 Amelogenin 86, 87 TMR-86 HUMvWFA31 76, 40 TMR-40 D8S1179 74, 75 TMR-75 HUMTPOX 72, 73 TMR-73 HUMFIBRA 70, 71 TMR-70 D5S818 84, 85 R6G-85 D13S317 82, 83 R6G-83 D7S820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-79 S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
Amelogenin 86, 87 TMR-86 HUMvWFA31 76, 40 TMR-40 D8S1179 74, 75 TMR-75 HUMTPOX 72, 73 TMR-73 HUMFIBRA 70, 71 TMR-70 D5S818 84, 85 R6G-85 D13S317 82, 83 R6G-83 D7S820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
D8S1179 74, 75 TMR-75 HUMTPOX 72, 73 TMR-73 HUMFIBRA 70, 71 TMR-70 D5S818 84, 85 R6G-85 D13S317 82, 83 R6G-83 D7S820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
HUMTPOX 72, 73 TMR-73 HUMFIBRA 70, 71 TMR-70 D5S818 84, 85 R6G-85 D13S317 82, 83 R6G-83 D7S820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
HUMFIBRA 70, 71 TMR-70 D5S818 84, 85 R6G-85 D13S317 82, 83 R6G-83 D7S820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
D5S818 84, 85 R6G-85 D13S317 82, 83 R6G-83 D7S820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
D13S317 82, 83 R6G-83 D7S820 80, 81 R6G-80 D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
D16S539 29, 79 R6G-79 HUMCSF1PO 77, 78 R6G-78 S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
HUMCSF1PO 77, 78 R6G-78 S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
S159 90, 91 R6G-91 4 D3S1358 68, 69 FL-69	
4 D3S1358 68, 69 FL-69	
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HUMTHO1 66, 67 FL-66	
D21S11 64, 65 FL-65	
D18S51 62, 63 FL-62	
G475 88, 89 FL-88	
Amelogenin 86, 87 TMR-86	
HUMvWFA31 76, 40 TMR-40 D8S1179 74, 75 TMR-75	
HUMTPOX 72, 73 TMR-73	
HUMFIBRA 70, 71 TMR-70	
D5S818 84, 85 FL-85	
D13S317 82, 83 FL-83	
D7S820 80, 81 FL-80	
D16S539 29, 79 FL-79 HUMCSF1PO 77, 78 FL-78	
S159 90, 91 FL-91	
5 D3S1358 68, 69 FL-69	
HUMTHO1 66, 67 FL-66	
D21S11 64, 65 FL-65	
D18S51 62, 63 FL-62 G475 88. 94 FL-94	
G475 88, 94 FL-94 Amelogenin 86, 87 TMR-86	
HUMvWFA31 76, 40 TMR-40	
D8S1179 74, 75 TMR-75	
HUMTPOX 72, 73 TMR-73	
HUMFIBRA 70, 71 TMR-70	
D5S818 84, 85 FL-85 D13S317 82, 83 FL-83	
D7S820 80, 81 FL-80	
D16S539 29, 79 FL-79	
HUMCSF1PO 77, 78 FL-78	
S159 95, 96 FL-96	
6 D3S1358 69, 106 FL-69 HUMTHO1 38, 103 FL-38	
D21S11 64, 65 FL-65	
D18S51 101, 102 FL-101	
S159 92, 93 FL-93	
Amelogenin 105, 87 TMR-105	
HUMvWFA31 76, 40 TMR-40	
D8S1179 104, 75 TMR-104 HUMTPOX 72, 73 TMR-72	+
HUMFIBRA 70, 107 TMR-72	
D5S818 84, 85 FL-85	
D13S317 3, 4 FL-4	
D7S820 80, 81 FL-80	
D16S539 29, 97 FL-29 HUMCSF1PO 77, 98 FL-98	
C221 99, 100 FL-99	
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Example 1

Fluorescent Detection of Multiplex Amplification of Loci D3S1358, HUMTH01, D21S11, D18S51, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, and HUMCSF1PO as detected with the ABI PRISM® 310 Genetic Analyzer

In this Example, a DNA template was amplified simulta- 10 neously at the individual loci D3S1358, HUMTH01, D21S11, D18S51, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, and HUMCSF1PO in a single reaction vessel. The PCR amplification was performed in 25 µl of 1× Gold ST*R Buffer (50 15 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂, 160 μ g/ml BSA and 200 μ M each of dATP, dCTP, dGTP and dTTP) using 1 ng template, and 3.25 U AmpliTaq Gold™ DNA Polymerase. A GeneAmp® PCR System 9600 (Perkin Elmer, Foster City, Calif.) was 20 employed with the following amplification protocol: 96° C. for 12 min., then 10 cycles of 94° C. for 30 sec., ramp for 68 sec. to 58° C., hold for 30 sec., ramp 50 sec. to 70° C., hold for 45 sec., followed by 20 cycles of 90° C. for 30 sec., ramp 60 sec. to 58° C., hold for 30 sec., ramp for 50 sec, to 25 70° C., hold for 45 sec., followed by 1 cycle of 60° C. for

Twenty-six amplification primers were used in combination, including 0.12 µM each D3S1358 primers 1 [SEQ ID HUMTH01 primers 1 [FL-SEQ ID NO:66] and 2 [SEQ ID NO:67], 0.3 μM each D21S11 primers 1 [SEQ ID NO:64] and 2 [FL-SEQ ID NO:65], 0.2 μ M each D18S51 primers 1 [FL-SEQ ID NO:62] and 2 [SEQ ID NO:63], 1.1 μ M each HUMvWFA31 primers 1 [SEQ ID NO:76] and 2 [TMR- 35 SEQ ID NO:40], 1.8 µM each D8S1179 primers 1 [SEQ ID NO:74] and 2 [TMR-SEQ ID NO:75], 0.6 μ M each HUMT-POX primers 1 [SEQ ID NO:72] and 2 [TMR-SEQ ID NO:73], 2.4 μM each HUMFIBRA primers 1 [TMR-SEQ ID NO:70] and 2 [SEQ ID NO:71], 0.2 μ M each D5S818 40 primers 1 [SEO ID NO:84] and 2 [R6G-SEO ID NO:85], 0.1M each D13S317 primers 1 [SEQ ID NO:82] and 2 [R6G-SEQ ID NO:83], 0.2 μ M each D7S820 primers 1 [R6G-SEQ ID NO:80] and 2 [SEQ ID NO:81], 0.15 μ M ID NO:79], 0.2 μM each HUMCSF1PO primers 1 [SEQ ID NO:77] and 2 [R6G-SEQ ID NO:78]

Amplified products were separated using an ABI PRISM® 310 Genetic Analyzer. DNA samples were mixed with 24 μ l of a loading solution (deionized formamide) and 50 $1.0 \,\mu$ l of an internal lane size standard, denatured at 95° C. for 3 min., and chilled on ice prior to injection. Separation was carried out using Performance Optimized Polymer 4 (POP-4)(Perkin Elmer Biosystems, Foster City, Calif.) in a 47 cm×50 µm capillary. The manufacturer's GeneScan® run 55 module GS STR POP4 (Id.) (1 ml) A was used. Conditions for the electrophoresis were a 5 second injection, injection kV was 15.0, run kV was 15.0, run temperature was 60° C., run time was 28 minutes and virtual filter A was used.

FIG. 1A is a printout of results of scanning the amplified 60 fragments of each locus separated and detected with the ABI PRISM® 310 Genetic Analyzer, as described above. FIG. 1A shows amplification products of a DNA sample simultaneously co-amplified for the loci D3S1358, HUMTH01, D21S11, D18S51, HUMvWFA31, D8S1179, HUMTPOX, 65 with 24 µl of a loading solution (deionized formamide) and HUMFIBRA, D5S818, D7S820, D13S317, D16S539, and HUMCSF1PO. Peaks shown in Panel A are labeled with

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fluorescein, peaks shown in Panel B are labeled with carboxy-tetramethylrhodamine, and peaks shown in Panel C are labeled with 5,6 carboxyrhodamine 6G.

FIG. 1B is a printout of the results of scanning a sample prepared in the same way as the sample scanned in FIG. 1A, except that no DNA template was used in the amplification reaction. Peaks in this figure are background products resulting from dye conjugation and purification procedures and from undefined causes.

Example 2

Fluorescent Detection of Multiplex Amplification of Loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO, and S159 as detected with the ABI PRISM® 310 Genetic Analyzer

In this Example, a DNA template was amplified simultaneously at the individual loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and S159 in a single reaction vessel. The PCR amplification was performed in 25 μl of 1× Gold ST*R Buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂, 160 μg/ml BSA and 200 μM each of dATP, dCTP, dGTP and NO:68] and 2 [FL-SEQ ID NO:69], 0.08 µM each 30 dTTP) using 1 ng template, and 4 U AmpliTaq Gold™ DNA Polymerase. A GeneAmp® PCR System 9600 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 12 min., then 10 cycles of 94° C. for 30 sec., ramp for 68 sec. to 58° C., hold for 30 sec., ramp 50 sec. to 70° C., hold for 45 sec., followed by 20 cycles of 90° C. for 30 sec., ramp 60 sec. to 58° C., hold for 30 sec., ramp for 50 sec, to 70° C., hold for 45 sec., followed by 1 cycle of 60° C. for 30 min.

Thirty-two amplification primers were used in combination, including 0.12 µM each D3S1358 primers 1 [SEQ ID NO:68] and 2 [FL-SEQ ID NO:69], 0.08 μM each HUMTH01 primers 1 [FL-SEQ ID NO:66] and 2 [SEQ ID NO:67], 0.3%µM each D21 S11 primers 1 [SEQ ID NO:64] and 2 [FL-SEQ ID NO:65], 0.2 μ M each D18S51 primers 1 each D16S539 primers 1 [SEQ ID NO:29] and 2 [R6G-SEQ 45 [FL-SEQ ID NO:62] and 2 [SEQ ID NO:63], 0.24 µM each G475 primers 1 [FL-SEQ ID NO:88] and 2 [SEQ ID NO:89], 0.6 μM each Amelogenin primers 1 [TMR-SEQ ID NO:86] and 2 [SEQ ID NO:87], $1.1 \mu M$ each HUMvWFA31 primers 1 [SEO ID NO:76] and 2 [TMR-SEO ID NO:40], 1.8 μ M each D8S1179 primers 1 [SEQ ID NO:74] and 2 [TMR-SEQ ID NO:75], 0.6 µM each HUMTPOX primers 1 [SEQ ID NO:72] and 2 [TMR-SEQ ID NO:73], 2.4 µM each HUMFIBRA primers 1 [TMR-SEQ ID NO:70] and 2 [SEQ ID NO:71], 0.2 μM each D5S818 primers 1 [SEQ ID NO:84] and 2 [R6G-SEQ ID NO:85], 0.1 µM each D13S317 primers 1 [SEQ ID NO:82] and 2 [R6G-SEQ ID NO:83], 0.2 μ M each D7S820 primers 1 [R6G-SEO ID NO:80] and 2 [SEO ID NO:81], 0.15 μ M each D16S539 primers 1 [SEQ ID NO:29] and 2 [R6G-SEQ ID NO:79], 0.2 µM each HUMCSF1PO primers 1 [SEQ ID NO:77] and 2 [R6G-SEQ ID NO:78] 0.1 μM each S159 primers 1 [SEQ ID NO:90] and 2 [R6G-SEQ ID NO:91]

> Amplified products were separated using an ABI PRISM® 310 Genetic Analyzer. DNA samples were mixed 1.0 µl of an internal lane size standard, denatured at 95° C. for 3 min., and chilled on ice prior to injection. Separation

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was carried out using Performance Optimized Polymer 4 (POP-4) (Perkin Elmer Biosystems, Foster City, Calif.) in a 47 cm×50 μm capillary. The manufacturer's GeneScan® run module GS STR POP4 (Id.)(1 ml) A was used. Conditions for the electrophoresis were a 5 second injection, injection 5 kV was 15.0, run kV was 15.0, run temperature was 60° C., run time was 28 minutes and virtual filter A was used.

FIG. 2A is a printout of results of scanning the amplified fragments of each locus separated and detected with the ABI PRISM® 310 Genetic Analyzer, as described above. FIG. 10 2A shows amplification products of a DNA sample simultaneously co-amplified for the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO, and S159. Peaks 15 shown in Panel A are labeled with fluorescein, peaks shown in Panel B are labeled with carboxy-tetramethylrhodamine, and peaks shown in Panel C are labeled with 5,6 carboxyrhodamine 6G.

FIG. 2B is a printout of the results of scanning a sample 20 prepared in the same way as the sample scanned in FIG. 2A, except that no DNA template was used in the amplification reaction. Peaks in this figure are background products resulting from dye conjugation and purification procedures and from undefined causes.

Example 3

Fluorescent Detection of Multiplex Amplification of Loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO, and S159 as detected with the ABI PRISM® 377 DNA Sequencer

In this Example, a DNA template was amplified as in Example 2. Amplified products were separated using an ABI PRISM® 377 DNA Sequencer. This was carried out using a 0.2 mm thick, 5% Long Ranger™ Acrylamide (FMC Bio-Products, Rockland, Me.), 7M urea gel. DNA samples were mixed with 1.51 µl of a loading solution (88.25% formamide, 4.1 mM EDTA, 15 mg/ml Blue Dextran) and 0.5 μ l of and chilled on ice prior to loading. Electrophoresis was carried out using the manufacturer's GeneScan® modules for Prerun (PR GS 36A-2400) and Run (GS 36A-2400). Run time was 3 hours and virtual filter A was used.

FIG. 3A is a printout of results of scanning the amplified $_{50}$ fragments of each locus separated and detected with the ABI PRISM® 377 DNA Sequencer, as described above. FIG. 3A shows amplification products of a DNA sample simultaneously co-amplified for the loci D3S1358, HUMTH01, D21S11, D 18S51, G475, Amelogenin, HUMvWFA31, 55 D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO, and S159. Peaks shown in Panel A are labeled with fluorescein, peaks shown in Panel B are labeled with carboxy-tetramethylrhodamine, and peaks shown in Panel C are labeled with 5,6 carbox- 60 yrhodamine 6G.

FIG. 3B is a printout of the results of scanning a sample prepared in the same way as the sample scanned in FIG. 3A, except that no DNA template was used in the amplification reaction. Peaks in this figure are background products result- 65 ing from dye conjugation and purification procedures and from undefined causes.

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Example 4

Fluorescent Detection of Multiplex Amplification of Loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO, and S159 as detected with the Hitachi FMBIO® II Fluorescent Scanner

In this example, two DNA templates were each amplified simultaneously at each of three different locus combinations selected from the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and S159, Amplification of each locus combination included 5 ng template in a single reaction vessel containing 25 μ l of 1× Gold ST*R Buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂, 160 μ g/ml BSA and 200 μ M each of dATP, dCTP, dGTP and dTTP).

A GeneAmp® PCR System 9600 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 12 min., then 10 cycles of 94° C. for 30 25 sec., ramp for 68 sec. to 58° C., hold for 30 sec., ramp 50 sec. to 70° C., hold for 45 sec., followed by 22 cycles of 90° C. for 30 sec., ramp 60 sec. to 58° C., hold for 30 sec., ramp for 50 sec, to 70° C., hold for 45 sec., followed by 1 cycle of 60° C. for 30 min.

Thirty-two amplification primers were used in the following concentrations, including 0.225 µM each D3S1358 primers 1 [SEQ ID NO:68] and 2 [FL-SEQ ID NO:69], 0.2 μM each HUMTH01 primers 1 [FL-SEQ ID NO:66] and 2 [SEQ ID NO:67], 1.0 µM each D21S11 primers 1 [SEQ ID 35 NO:64] and 2 [FL-SEQ ID NO:65], 1.0 μM each D18S51 primers 1 [FL-SEQ ID NO:62] and 2 [SEQ ID NO:63], 2.8 μM each G475 primers 1 [FL-SEQ ID NO:88] and 2 [SEQ ID NO:89], 0.2 μM each Amelogenin primers 1 [TMR-SEQ ID NO:86] and 2 [SEQ ID NO:87], 0.3 μ M each HUMvWFA31 primers 1 [SEQ ID NO:76] and 2 [TMR-SEQ ID NO:40], 1.5 μM each D8S1179 primers 1 [SEQ ID NO:74] and 2 [TMR-SEQ ID NO:75], 0.2 µM each HUMTPOX primers 1 [SEQ ID NO:72] and 2 [TMR-SEQ ID NO:73], 2.0 µM each HUMFIBRA primers 1 [TMR-SEQ ID NO:70] an internal lane size standard, denatured at 95° C. for 2 min., 45 and 2 [SEQ ID NO:71], 0.55 µM each D5S818 primers 1 [SEQ ID NO:84] and 2 [FL-SEQ ID NO:85], 1.1/M each D13S317 primers 1 [SEQ ID NO:82] and 2 [FL-SEQ ID NO:83], 1.7 μ M each D7S820 primers 1 [FL-SEQ ID NO:80] and 2 [SEQ ID NO:81], 3.3 µM each D16S539 primers 1 [SEQ ID NO:29] and 2 [FL-SEQ ID NO:79], 0.5 μM each HUMCSF1PO primers 1 [SEQ ID NO:77] and 2 [FL-SEQ ID NO:78], 2.0 μM each S159 primers 1 [SEQ ID NO:90] and 2 [FL-SEQ ID NO:91].

> In the first locus combination, each template was amplified using 2.5 U of AmpliTaq Gold™ DNA Polymerase and primers for each locus used in the concentrations described above for the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, and HUMFIBRA. In the second locus combination, all thirty-two primers, above, at the described concentrations, and 4 U of AmpliTaq Gold™ DNA Polymerase were used to amplify DNA templates at all sixteen loci, D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and S159 in a single reaction vessel. In the third combination, each template was amplified using 1.5 U of AmpliTaq Gold™ DNA

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Polymerase and primers for each locus used in the concentrations described above for the loci D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and S159.

Amplification products were separated by electrophoresis through a 0.4 mm thick 4% denaturing polyacrylamide gel (19:1 ratio of acrylamide to bis-acrylamide) which contained 7 M urea (Sambrook et al., (1989)), and which was chemically cross-linked to 2 glass plates (Kobayashi, Y. (1988), BRL Focus 10: 73-74). DNA samples were mixed with 3.5 μ l, of a loading solution (10 mM NaOH, 95% formamide, 10 0.05% bromophenol blue) and $0.5 \mu l$ of an internal lane size standard, denatured at 95° C. for 2 min., and chilled on ice prior to loading. The separated products were visualized by detection of the fluorescent signals using the Hitachi FMIBO® II fluorescent scanner (Hitachi Software Engineering America, Ltd. South San Francisco, Calif.). Band pass filters at 505 nm and 585 nm, respectively, were used for the detection of fluorescein-labeled loci and carboxytetramethylrhodamine-labeled loci, respectively. A band pass filter of 650 nm was used for detection of the internal lane standard (size standard data, not shown).

Reference is made to FIGS. 4A and 4B, which display the fragments resulting from each amplification reaction. FIG. 4A shows the results from the 505 nm scan (Fluorescein channel) and FIG. 4B shows the results from the 585 nm scan (carboxy-tetramethylrhodamine channel) of the same 25 lanes of the polyacrylamide gel. For each DNA template, lane 1 shows the results of the DNA sample which has been simultaneously co-amplified for the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, and HUMFIBRA. Lane 2 30 shows the results of the DNA sample simultaneously coamplified for the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D13S317, D7S820, D16S539, HUMCSF1PO, and S159. Lane 3 shows the 35 results of the DNA sample simultaneously co-amplified for the loci D5S818, D13S317, D7S820, D16S539, HUMCSF1PO, and S159.

Example 5

Fluorescent Detection of Multiplex Amplification of Loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO, and S159 as detected with the Hitachi FMBIO® II Fluorescent

In this example, two DNA templates were each amplified 50 simultaneously at each of two different locus combinations selected from the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and S159. Amplification of each locus combination included 5 ng template in a single reaction vessel containing 25 µl of 1× Gold ST*R Buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂ 160μ g/ml BSA and 200μ M each of dATP, dCTP, dGTP and dTTP).

A GeneAmp® PCR System 9600 (Perkin Elmer, Foster 60 City, Calif.) was employed with the following amplification protocol: 96° C. for 12 min., then 10 cycles of 94° C. for 30 sec., ramp for 68 sec. to 58° C., hold for 30 sec., ramp 50 sec. to 70° C., hold for 45 sec., followed by 22 cycles of 90° C. 50 sec, to 70° C., hold for 45 sec., followed by 1 cycle of 60° C. for 30 min.

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Thirty-two amplification primers were used in the following concentrations, including 0.225 µM each D3S1358 primers 1 [SEQ ID NO:68] and 2 [FL-SEQ ID NO:69], 0.2 μM each HUMTH01 primers 1 [FL-SEQ ID NO:66] and 2 [SEQ ID NO:67], 1.0 µM each D21S111 primers 1 [SEQ ID NO:64] and 2 [FL-SEQ ID NO:65], 1.0 μM each D18S51 primers 1 [FL-SEQ ID NO:62] and 2 [SEQ ID NO:63], 2.8 μ M each G475 primers 1 [SEQ ID NO:88] and 2 [FL-SEQ ID NO:94], 0.2 μM each Amelogenin primers 1 [TMR-SEQ ID NO:86] and 2 [SEQ ID NO:87], 0.3 µM each HUMvWFA31 primers 1 [SEQ ID NO:76] and 2 [TMR-SEQ ID NO:40], 1.5 μ M each D8S1179 primers 1 [SEQ ID NO:74] and 2 [TMR-SEQ ID NO:75], 0.2 μ M each HUMTPOX primers 1 [SEQ ID NO:72] and 2 [TMR-SEQ ID NO:73], 2.0 µM each HUMFIBRA primers 1 [TMR-SEQ ID NO:70] and 2 [SEQ ID NO:71], 0.55 μ M each D5S818 primers 1 [SEQ ID NO:84] and 2 [FL-SEQ ID NO:85], 1.1 μ M each D13S317 primers 1 [SEQ ID NO:82] and 2 [FL-SEQ ID NO:83], 1.7 μ M each D7S820 primers 1 [FL-SEQ ID NO:80] and 2 [SEQ ID NO:81], 3.3 μ M each D16S539 primers 1 [SEQ ID NO:29] and 2 [FL-SEQ ID NO:79], 0.5 µM each HUMCSF1PO primers 1 [SEQ ID NO:77] and 2 [FL-SEQ ID NO:78], $2.0\,\mu\text{M}$ each \$159 primers 1 [SEQ ID NO:95] and 2 [FL-SEQ ID NO:96].

In the first locus combination, each template was amplified using 2.5 U of AmpliTaq Gold™ DNA Polymerase and primers for each locus used in the concentrations described above for the loci D3S1358, HUMTH01, D21S11, D18S51. G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, and HUMFIBRA. In the second locus combination, all thirty-two primers, above, at the described concentrations, and 4 U of AmpliTaq Gold™ DNA Polymerase were used to amplify DNA templates at all sixteen loci, D3S1358, HUMTH01, D21 S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and S159 in a single reaction vessel.

The separation and visualization of amplified products were as described in Example 4.

Reference is made to FIGS. 5A and 5B, which display the fragments resulting from each amplification reaction. FIG. 5A shows the results from the 505 nm scan (Fluorescein channel) and FIG. 5B shows the results from the 585 nm scan (carboxy-tetramethylrhodamine channel) of the same lanes of the polyacrylamide gel. For each template, lane 1 shows the results of the DNA sample simultaneously co-45 amplified for the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, and HUMFIBRA and lane 2 shows the results of the DNA sample simultaneously co-amplified for the loci D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D13S317, D7S820, D16S539, HUMCSF1PO, and S159.

Example 6

Fluorescent Detection of Multiplex Amplification of Loci D3S1358, HUMTH01, D21S11, D18S51, S159, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO, and C221 as detected with the Hitachi FMBIO® II Fluorescent Scanner

In this example, two DNA templates were each amplified for 30 sec., ramp 60 sec. to 58° C., hold for 30 sec., ramp for 65 simultaneously at each of three different locus combinations selected from the loci D3S1358, HUMTH01, D21S11, D18S51, S159, Amelogenin, HUMvWFA31, D8S1179,

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HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and C221. Amplification of each locus combination included 10 ng template in a single reaction vessel containing 25 μ l of 1× Gold ST*R Buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25° C.), 0.1% Triton 5 X-100, 1.5 mM MgCl₂, 160 μ g/ml BSA and 200 μ M each of dATP, dCTP, dGTP and dTTP).

A GeneAmp® PCR System 9600 (Perkin Elmer, Foster City, Calif.) was employed with the following amplification protocol: 96° C. for 12 min., then 10 cycles of 94° C. for 30 10 sec., ramp for 68 sec. to 60° C., hold for 30 sec., ramp 50 sec. to 70° C., hold for 45 sec., followed by 20 cycles of 90° C. for 30 sec., ramp 60 sec. to 60° C., hold for 30 sec., ramp for 50 sec, to 70° C., hold for 45 sec., followed by 1 cycle of 60° C. for 30 min.

Thirty-two amplification primers were used in the following concentrations, including $0.75 \mu M$ each D3S1358 primers 1 [SEQ ID NO:106] and 2 [FL-SEQ ID NO:69], 0.3 μM each HUMTH01 primers 1 [FL-SEQ ID NO:38] and 2 [SEQ ID NO:103], 2.0 μ M each D21S11 primers 1 [SEQ ID 20 NO:64] and 2 [FL-SEQ ID NO:65], 0.3 µM each D18S51 primers 1 [FL-SEQ ID NO:101] and 2 [SEQ ID NO:102], $2.0~\mu M$ each S159 primers 1 [SEQ ID NO:92] and 2 [FL-SEQ ID NO:93], 0.15 μM each Amelogenin primers 1 [TMR-SEQ ID NO:105] and 2 [SEQ ID NO:87], 1.0 µM 25 each HUMvWFA31 primers 1 [SEQ ID NO:76] and 2 [TMR-SEQ ID NO:40], 1.25 µM each D8S1179 primers 1 [TMR-SEQ ID NO:104] and 2 [SEQ ID NO:75], 0.75 μ M each HUMTPOX primers 1 [TMR-SEQ ID NO:72] and 2 [SEQ ID NO:73], 1.5 µM each HUMFIBRA primers 1 30 [TMR-SEQ ID NO:70] and 2 [SEQ ID NO:107], 0.55 μ M each D5S818 primers 1 [SEQ ID NO:84] and 2 [FL-SEQ ID NO:85], 1.1 μM each D13S317 primers 1 [SEQ ID NO:3] and 2 [FL-SEQ ID NO:4], 1.7 μ M each D7S820 primers 1 [FL-SEQ ID NO:80] and 2 [SEQ ID NO:81], $3.3 \mu M$ each 35 D16S539 primers 1 [FL-SEQ ID NO:29] and 2 [SEQ ID NO:97], 0.25 μM each HUMCSF1PO primers 1 [SEQ ID NO:77] and 2 [FL-SEQ ID NO:98], 1.0 µM each C221 primers 1 [FL-SEQ ID NO:99] and 2 [SEQ ID NO: 100].

In the first locus combination, each template was amplified using 2.5 U of AmpliTaq Gold™ DNA Polymerase and primers for each locus used in the concentrations described

above for the loci D3S1358, HUMTH01, D21 S11, D18S51, S159, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, and HUMFIBRA. In the second locus combination, all thirty-two primers, above, at the described concentrations and 4 U of AmpliTaq Gold™ DNA Polymerase were used to amplify DNA templates at all sixteen loci, D3S1358, HUMTH01, D21S11, D18S51, S159, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and C221 in a single reaction vessel. In the third combination, each tem-

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single reaction vessel. In the third combination, each template was amplified using 1.5 U of AmpliTaq Gold™ DNA Polymerase and primers for each locus used in the concentrations described above for the loci D5S818, D7S820, D13S317, D16S539, HUMCSF1PO and C221.

The amplification products were separated and detected as described in Example 4, except that each sample of amplification products was diluted 1:4 in 1× STR Buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25° C.), 0.1% Triton X-100, 1.5 mM MgCl₂, and 200 μ M each of dATP, dCTP, dGTP and dTTP). The diluted amplification products (2.5 μ) were mixed with 2.5 μ l of a loading solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue), without an internal lane standard, denatured at 95° C. for 2 min., and chilled on ice prior to loading.

Reference is made to FIGS. 6A and 6B, which display the fragments resulting each amplification reaction. FIG. 6A shows the results from the 505 nm scan (Fluorescein channel) and FIG. 6B shows the results from the 585 nm scan (carboxy-tetramethylrhodamine channel) of the same lanes of the polyacrylamide gel. For each DNA template, lane 1 shows the results of the DNA sample simultaneously coamplified for the loci D3S1358, HUMTH01, D21S11, D18S51, S159, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, and HUMFIBRA. Lane 2 shows the results of the DNA sample simultaneously co-amplified for the loci D3S1358, HUMTH01, D21 S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D13S317, D7S820, D16S539, HUMCSF1PO, and C221. Lane 3 shows the results of the DNA sample simultaneously co-amplified for the loci D5S818, D13S317, D7S820, D16S539, HUMCSF1PO, and C221.

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51

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55 56

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57 58

-continued	
agaaagaaaa aaaagaaaga aaagaaaaga aaagaaaaga	500
aaagaaaaga aaaaacgaag gggaaaaaaa gagaatcata aacataaatg	550
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catcaaaact taaaagttct actcttcaaa agatacctta taaagaaagt	700
aaaaagacac gccacaggct aagagaaagt acttctaatc acatatctaa	750
aaaaggactt gtgtccagat taaagaattc ttacacatca ataagacaac	800
ccaattaaaa atcggcaaaa gatttgaaga gatatttaac caaagaaaac	850
atataaatgt gtccgggcgc gatggtaatc ccagcacttt gagaggccga	900
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aagagggact aagttttgtt ttgttttgtt ttgttttgtt	200
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tcagtgttgg aatgctctct tgtagcagtg gcggctgctg ctggttccgg	300
gtcagatgcc ggaattgggg gtgcgcttgg gtgcagctgc atttcatctg	350
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aattotttoo atgtatoaat catgatacta agcactttac acacatgtat	200
gttatgtaat cattatatca tgcatgcaag gtaatgagta ttattttcct	250
cattttataa aagaggaaac tgatgtttga ggctactttg cttaagaccg	300
cagaactagc aaaggaaaag agaagtgaat gtatc	335

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What is claimed is:

- 1. A kit for simultaneously analyzing a set of loci of genomic DNA, comprising oligonucleotide primers for coamplifying a set of loci of the genomic DNA to be analyzed, wherein the primers are in one or more containers, wherein the primers are designed to co-amplify a set of at least sixteen loci which can be co-amplified, comprising
 - D3S1358, HUMTH01, D21S11, D18S51, G475, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, 10 HUMCSF1PO, and S159.
- 2. A kit for simultaneously analyzing a set of loci of genomic DNA, comprising oligonucleotide primers for coamplifying a set of loci of the genomic DNA to be analyzed, wherein the primers are in one or more containers, wherein 15 the primers are designed to co-amplify a set of at least sixteen loci which can be co-amplified, comprising D3S1358, HUMTH01, D2IS11, D18S51, S159, Amelogenin, HUMvWFA31, D8S1179, HUMTPOX, HUMFIBRA, D5S818, D7S820, D13S317, D16S539, HUMCSF1PO, and 20 C221
- 3. The kit of claim 1, wherein at least one oligonucleotide primer has a sequence selected from one of the groups of primer sequences consisting of:
 - SEQ ID NO:68, SEQ ID NO:69, and SEQ ID NO:106, for 25 the locus D3S1358;
 - SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:66, SEQ ID NO:67, and SEQ ID NO:103, for the locus HUMTH01;
 - SEQ ID NO:64 and SEQ ID NO:65, for the locus D21S11;
 - SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:101, and SEQ ID NO:102, for the locus D18S51;
 - SEQ ID NO:88, SEQ ID NO:89, and SEQ ID NO:94, for the locus G475;
 - SEQ ID NO:86, SEQ ID NO:87, and SEQ ID NO:105, for the Amelogenin locus;
 - SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:59, SEQ ID NO:60, and SEQ ID NO:76, for the locus HUM-vWFA31;
 - SEQ ID NO:74, SEQ ID NO:75, and SEQ ID NO:104, for the locus D8S1179;
 - SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:72, and SEQ ID NO:73, for the locus HUMTPOX;
 - SEQ ID NO:70, SEQ ID NO:71, and SEQ ID NO:107, for the locus HUMFIBRA;

 SEQ ID NO:62, SEQ ID NO:62, SEQ ID NO:62, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:64, SEQ ID NO:64, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:662, SEQ
 - SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:84, and SEQ ID NO:85, for the locus D5S818;
 - SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:80, and SEQ ID NO:81, for the locus D7S820;
 - SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:82, and SEQ ID NO:83, for the locus D13S317;
 - SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:58, SEQ ID NO:79, and SEQ ID NO:97, for the locus D16S539;
 - SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:77, SEQ ID NO:78, and SEQ ID NO:98, for the locus HUMCSF1PO; and
 - SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:95, and SEQ ID NO:96, for the locus \$159
- 4. The kit of claim 2, wherein at least one oligonucleotide primer has a sequence selected from one of the groups of primer sequences consisting of:
 - SEQ ID NO:68, SEQ ID NO:69, and SEQ ID NO:106, for the locus D3S1358;
 - SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:66, SEQ ID NO:67, and SEQ ID NO:103, for the locus HUMTH01;

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- SEQ ID NO:64 and SEQ ID NO:65, for the locus D21S11;
- SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:101, and SEQ ID NO:102, for the locus D18S51;
- SEQ ID NO:88, SEQ ID NO:89, and SEQ ID NO:94, for the locus S159;
- SEQ ID NO:86, SEQ ID NO:87, and SEQ ID NO:105, for the Amelogenin locus;
- SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:59, SEQ ID NO:60, and SEQ ID NO:76, for the locus HUM-vWFA31;
- SEQ ID NO:74, SEQ ID NO:75, and SEQ ID NO:104, for the locus D8S1179;
- SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:72, and SEQ ID NO:73, for the locus HUMTPOX;
- SEQ ID NO:70, SEQ ID NO:71, and SEQ ID NO:107, for the locus HUMFIBRA;
- SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:84, and SEQ ID NO:85, for the locus D5S818;
- SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:80, and SEQ ID NO:81, for the locus D7S820;
- SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:82, and SEQ ID NO:83, for the locus D13S317;
- SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:58, SEQ ID NO:79, and SEQ ID NO:97, for the locus D16S539;
- SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:77, SEQ ID NO:78, and SEQ ID NO:98, for the locus HUMCSF1PO; and
- SEQ ID NO:99 and SEQ ID NO:100, for the locus C221.
- 5. A kit for simultaneously analyzing a set of loci of genomic DNA, comprising oligonucleotide primers for coamplifying a set of loci of the genomic DNA to be analyzed, wherein the primers are in one or more containers, wherein the primers are designed to co-amplify a set of loci from one or more DNA samples, comprising short tandem repeat loci D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, HUMCSF1PO, HUMFIBRA, HUMTH01, HUMTPOX, HUMvWFA31, and a locus selected from the group consisting of G475, S159, C221, and Amelogenin.
- 6. The kit of claim 5, wherein at least one oligonucleotide primer has a sequence selected from one of the groups of primer sequences consisting of:
 - SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:101, and SEQ ID NO:102, when one of the loci in the set is D18S51;
 - SEQ ID NO:64 and SEQ ID NO:65, for the locus D21S11;
 - SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:66, SEQ ID NO:67, and SEQ ID NO:103, for the locus HUMTH01;
 - SEQ ID NO:68, SEQ ID NO:69, and SEQ ID NO:106, for the locus D3S1358;
 - SEQ ID NO:70, SEQ ID NO:71, and SEQ ID NO:107, for the locus HUMFIBRA;
 - SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:72, and SEQ ID NO:73, for the locus HUMTPOX;
 - SEQ ID NO:74, SEQ ID NO:75, and SEQ ID NO:104, for the locus D8S1179;
 - SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:59, SEQ ID NO:60, and SEQ ID NO:76, for the locus HUM-vWFA31;
 - SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:77, SEQ ID NO:78, and SEQ ID NO:98, for the locus HUMCSF1PO;

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SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:58, SEQ ID NO:79, and SEQ ID NO:97, for the locus D16S539;

SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:80, and SEQ ID NO:81, for the locus D7S820;

SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:82, and SEQ 5 ID NO:83, for the locus D13S317;

SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:84, and SEQ ID NO:85, for the locus D5S818;

SEQ ID NO:88, SEQ ID NO:89, and SEQ ID NO:94, for the locus G475;

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SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:95, and SEQ ID NO:96, for the locus S159:

SEQ ID NO:99 and SEQ ID NO:100, for the locus C221; and

SEQ ID NO:86, SEQ ID NO:87, and SEQ ID NO:105, for the Amelogenin locus.

* * * * *

Case: 13-1011 Cast SE-PARTICIPANTS CON 88 DORANGE 13:373 FIRM 607332/2013 ed: 07/12/2013

UNITED STATES PATENT AND TRADEMARK OFFICE CERTIFICATE OF CORRECTION

PATENT NO. : 7,008,771 B1 Page 1 of 4

APPLICATION NO.: 10/236577 DATED: March 7, 2006

INVENTOR(S) : James W. Schumm et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On The Title Page, Item -56-

In (56) References Cited, U.S. PATENT DOCUMENTS, after U.S. patent "5,843,660 A, 12/1998, Schumm et al.," insert --6,013,444 A, 1/2000, Dau et al.--.

On The Title Page, Item -56-

In (56) References Cited, FOREIGN PATENT DOCUMENTS, after foreign patent "DE 38 34 636 C2, 4/1990," insert --EP 0 846 775, 6/1998--.

On The Title Page, Item -56-

In (56) References Cited, FOREIGN PATENT DOCUMENTS, after foreign patent "WO 96/10648, 4/1996," insert --WO 97/39138, 10/1997--.

On The Title Page, Item -56-

In (56) References Cited, OTHER PUBLICATIONS, after reference "Niezgoda, Stephen J. Jr., et al., "The FBI Laboratory's Combined DNA Index System Program," (1995) The Sixth International Symposium on Human Identification, pp. 149-153," insert the following references:

--Amiott, et al. "Incorporating high quality markers into forensically useful mulitplexes," Human Identification Symposium Proceedings: 9th International Symposium on Human Identification, October 8-10, 1998, pp. 2-6.

Lins, A. et al., "Development and population study of an eight-locus short tandem repeat (STR) multiplex system," J. of Forensic Sciences, (Nov. 1998) 43(6) 1168-80.

Lin, Z. et al., "Multiplex genotype determination at a large number of gene loci," Proceedings of the National Academy of Sciences of the United States of America, (March 19, 1996) 93(6) pp. 2582-7.

McKeown B., et al. "Increasing the size of PCR products without redesigning primer building sequences," Nucleic Acids Research, (June 25, 1995) pp. 2337-8.

Oldroyd et al., "A highly discriminating octoplex short tandem repeat polymerase chain reaction suitable for human individual identification," Electrophoresis Vol. 16, pp. 334-337.

Schumm et al., "Pentanucleotide repeats: Highly polymorphic genetic markers displaying minimal stutter artifact," Human Identification Symposium Proceedings: 9th International Symposium on Human Identification, October 8-10, 1998, pp. 24-37.

UNITED STATES PATENT AND TRADEMARK OFFICE CERTIFICATE OF CORRECTION

PATENT NO. : 7,008,771 B1 Page 2 of 4

APPLICATION NO.: 10/236577 DATED: March 7, 2006

INVENTOR(S) : James W. Schumm et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Sparkes, R. et al. "The validation of a 7-locus multiplex STR test for use in forensic casework (II), Artifacts, casework studies and success rates," International J. Legal Medicine (1996) 109(4) pp. 195-204.

"The 9th international symposium on human identification October 8-10, 1998," Profiles in DNA, (Jan. 1999) Vol. 2 No. 3, p. 13 and Table of Contents.--

In (56) References Cited, OTHER PUBLICATIONS:

Page 2, after the Bever, Robert A. et al. reference, "Budowie" should read --Budowle--.

Page 3, under the Hudson, Thomas et al. reference, "Chromosmal" should read --Chromosomal--.

Page 3, under the Kimpton, C. et al. reference, "progiling" should read --profiling--.

Page 3, under the Lohmann, D. et al. reference, "retinolastoma" should read --retinoblastoma--.

Page 3, under the Pftizinger, Helene et al. reference, "Systemns" should read --Systems--.

Page 3, under the Richard, Malanie et al. reference, delete second occurrence of "Repeat (STR)"; "THO01" should read --TH01--; "Flyorescence" should read --Fluorescence--; "Fight" should read --Fifth--

Page 3, under the second Schumm, James W. et al. reference, "help" should read --held--.

Page 3, under the Shuber, Anthony reference, "PCPs" should read --PCRs--.

Page 4, line 1, insert --Symposium-- between 'International' and 'on'

Page 4, under the Williamson, R. et al. reference, "Cenet" should read --Genet--.

Page 4, under the McCabe, E.R. reference, "Sports" should read --Spots--.

UNITED STATES PATENT AND TRADEMARK OFFICE CERTIFICATE OF CORRECTION

PATENT NO. : 7,008,771 B1 Page 3 of 4

APPLICATION NO.: 10/236577 DATED: March 7, 2006

INVENTOR(S) : James W. Schumm et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Page 4, under the second Perkin-Elmer Corporation reference, "(1997)" should read --(1998)--; the first "pp." should be deleted.

Page 4, line 1, "Interneational" should read --International--.

Page 4, under the Niezgoda, S.J. reference, "(1997)" should read --(1998)--.

Page 4, after the Werrett, D.J. et al. reference, "Budowie" should read --Budowle--.

Page 5, under the Puers, C. et al. reference, "HUMTH01[AATG]," should read --HUMTH01[AATG] $_n$ --.

Page 5, under the Chen, H. et al. reference, "(ATTTT)," should read $--(ATTTT)_n$ --.

Page 5, line 2, "Glucocirticoid" should read --Glucocorticoid--.

Page 5, under the Litt, M. et al. reference, "Virto" should read -- Vitro--.

Column 1, line 37, delete "a" between 'at' and 'crime'.

Column 5, lines 14 and 15, delete "In another emobidment of the invention, the set of loci selected in step (b) of".

Column 8, line 60, " $(A_2G_xT_vC_7)_n$ " should read -- $(A_wG_xT_vC_7)_n$ --.

Column 15, line 6, "1 3.57" should read --13.57--.

Column 15, line 7, delete "for" between 'conditions' and 'suitable'.

Column 19, line 42, "0.1M" should read --0.1μM--.

Column 20, line 43, "0.3%μM" should read --0.3μM--.

Column 22, line 46, "1.1M" should read -1.1μ M--.

Case: 13-1011 Casas SB-PARTICIPANTS COINBY DOR 2018 13433 FIRED 107342/2018 ed: 07/12/2013

UNITED STATES PATENT AND TRADEMARK OFFICE CERTIFICATE OF CORRECTION

PATENT NO. : 7,008,771 B1 Page 4 of 4

APPLICATION NO. : 10/236577 DATED : March 7, 2006

INVENTOR(S) : James W. Schumm et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 26, line 21, " 2.5μ " should read -- 2.5μ l--.

Signed and Sealed this

Eighth Day of April, 2008

JON W. DUDAS
Director of the United States Patent and Trademark Office

Case: 13-1011 CaseASE-PARITICIPANTINGEDINBY DorangeenSt433 Filearty e073412/20163ed: 07/12/2013

TAB 10

(19) United States

(12) Reissued Patent

Jäckle et al.

US RE37,984 E (10) Patent Number:

(45) Date of Reissued Patent: Feb. 11, 2003

(54) PROCESS FOR ANALYZING LENGTH POLYMORPHISMS IN DNA REGIONS

- (75) Inventors: Herbert Jäckle, Göttingen (DE); Diethard Tautz, Köln (DE)
- (73) Assignee: Max-Planck-Gesellschaft zur

Forderung der Wissenschaften e.V.,

Gottingen (DE)

- (21) Appl. No.: 09/591,383
- (22) Filed: Jun. 9, 2000

Related U.S. Patent Documents

Reissue of:

5,766,847 (64) Patent No.: Jun. 16, 1998 Issued: 08/145,617 Appl. No.: Nov. 4, 1993 Filed:

U.S. Applications:

(63) Continuation of application No. 07/681,494, filed as application No. PCT/EP89/01203 on Oct. 11, 1989, now aban-

(30)Foreign Application Priority Data

Oct.	11, 1988	(DE)
(51)	Int. Cl. ⁷	C12P 19/34 ; C12Q 1/68;
		C07H 21/04
(52)	HS CL	435/6: 435/104: 435/01 02:

- 536/23.1; 536/24.3; 536/24.33
- **Field of Search** 435/6, 91.2, 194, 435/810; 536/22.1, 23.1, 24.3, 24.22

References Cited (56)

U.S. PATENT DOCUMENTS

5,075,217 A	12/1991	Weber	435/6
5,364,759 A	11/1994	Caskey et al	435/6

FOREIGN PATENT DOCUMENTS

EP	0 186271	10/1985
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(57)ABSTRACT

A process for analyzing length polymorphism in DNA regions wherein the following steps are carried out:

- (a) annealing at least one primer pair to the DNA to be analyzed, wherein one of the molecules of the primer pair is substantially complementary to one of the complementary strands of the 5' or 3' flank of a simple or cryptically simple DNA sequence, and wherein the annealing occurs in such an orientation that the synthesis products obtained by a primer-controlled polymerisation reaction with one of said primers can serve as template for annealing the other primer after dena-
- (b) primer-controlled polymerase chain reaction; and
- (c) separating and analyzing the polymerase chain reaction products.

41 Claims, 6 Drawing Sheets

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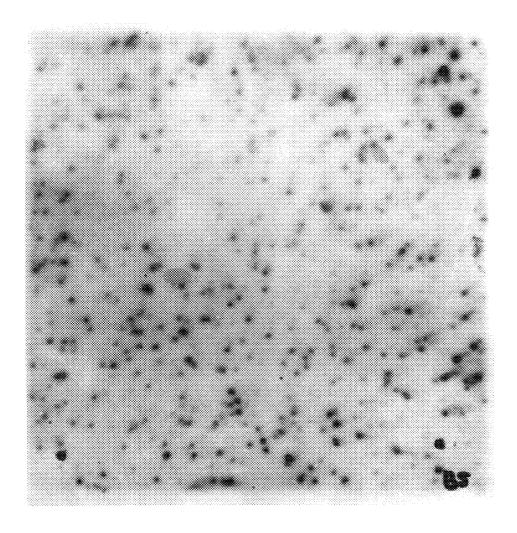


FIG.1

TAAGCTTGGGAATCATCTCGCCGACGGCCAGCGATATGGGCATCATGCTCGCCCCCCCC
AATCCTCGAAGAATAGTGCAATAATGCAAACGATATCACCCC <u>AGCAACAGCAGCAGCAGC</u>
AGCAGCAGCAACAGCAGCAACATCAGCAGCAGCAACAGCAGCAGCAACAGCAGCAGCAGCAG
202 nt 1 177 nt
<u>AACAGCAGCAA</u> CTCGGAGCCTGGAGTTCGGTTCAGAGGGCTTGGACCTGAATGGAT
Hae Ill
TTTGTGGATCTCCGGGTAAGTGGTCACTCATGATGGACTCTATGGACTCGCTAACTAGCT
AACTAATCATTCTACCATCCCAACTTGCAGACTCATTTCACTCGGGTCAAATGAATCCGC
CCTCGATACAAAGTTCAAT
(SEQ ID NO:5)

FIGURE 2

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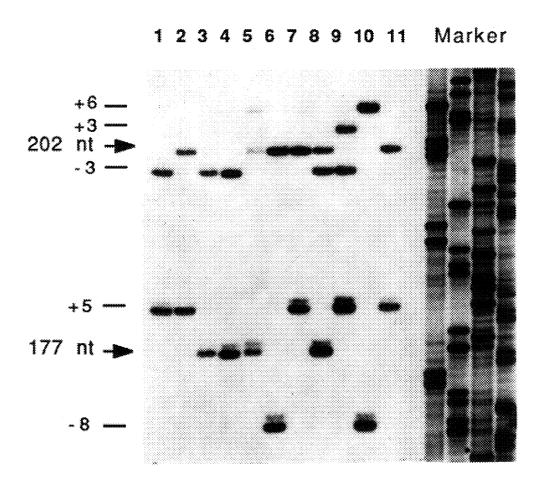


FIG.3

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PREPARATION A PREPARATION B 12345678910 12345678910 Marker

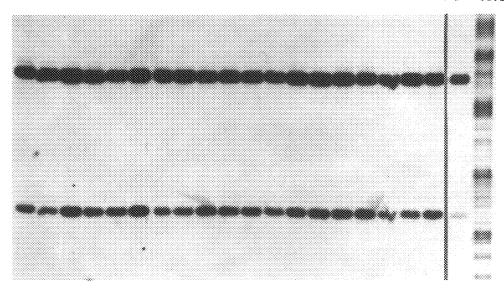


FIG.4

Case: 13-1011 CaseASB-PEARITICIPANTINSeOtN BY Dorcaugnee 1814833 Fileady e073428/201839ed: 07/12/2013

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5'-CTCCCCACACAAAGAAGTTCTGTTCTCTTCCCTCTACCTTGAT
GAATGCACTGTGA (TG)₁₅₋₂₅AC<u>TCGTTCCCAGGTATGGAA</u>-3'
(SEQ ID NO:6)

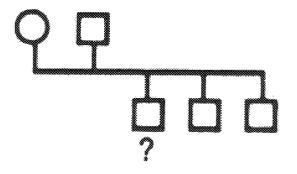
FIGURE 5

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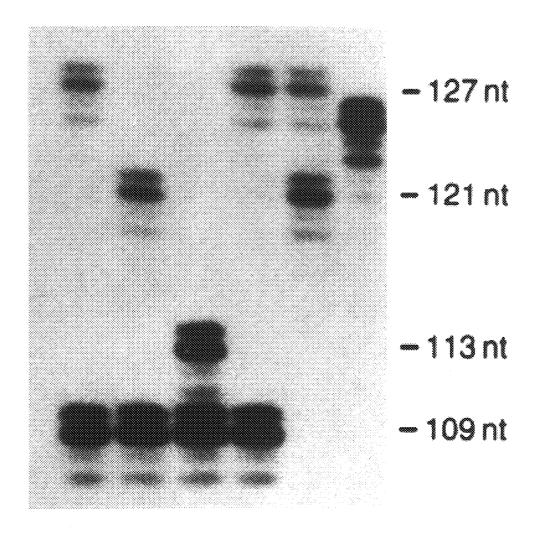


FIG.6

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PROCESS FOR ANALYZING LENGTH POLYMORPHISMS IN DNA REGIONS

Matter enclosed in heavy brackets [] appears in the original patent but forms no part of this reissue specifi- 5 cation; matter printed in italics indicates the additions made by reissue.

This application is a reissue application of U.S. Pat. No. 5,766,847, issued Jun. 16, 1998 from application Ser. No. 08/145,617, filed Nov. 4, 1993, which is a continuation of 10 [application Ser.] application No. 07/681,494 filed on Jun. 10, 1991, now abandoned. Application Ser. No. 07/681,494 is the national stage under 35 U.S.C. §371 of International Application PCT/EP89/01203, filed Oct. 11, 1989 and designating the United States.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to a process for determining identity 20 and kinship of organisms on the basis of length polymorphisms in the regions of simple or cryptically simple DNA sequences.

2. Description of Related Art

All usual processes for the determination of identity and 25 kinship on the basis of DNA length polymorphisms are based on the use of restriction endonucleases. Thereby specific DNA fragments are prepared which are afterwards detected by means of hybridization methods. With these methods either variations in length which have formed 30 between the corresponding recognition sites for restriction endonucleases or variations in length which, have formed due to the lack of certain restriction cleavage sites are analyzed. The first type of polymorphism analysis reveals the variation in length in so-called minisatellite regions (3, 4, 4a, 4b) and/or in regions with specific simple DNA sequences (5). The second analysis in which restriction fragment length polymorphisms (RFLP) due to the presence or absence of a restriction site, are detected can be applied only in specific, empirically found cases and can substantially be used appropriately only in the analysis of genetic

The disadvantage of both known methods lies in the fact that a hybridization reaction has to be carried out to make the length polymorphic regions visible. This makes the methods time-consuming and expensive. Furthermore, a single analysis using the previous methods does normally not allow any definitive conclusion about the relationship of two samples to be made so that additionally a second independent analysis becomes necessary. Therefore, these processes are not very appropriate for serial examinations and routine testing. Furthermore, the described method are not suitable for automation.

Higuchi et al. (5a) describe a further process for analyzing 55 a length polymorphic locus, comprising a primer-controlled polymerization reaction of certain mitochondrial DNA sequences. This process cannot be used for paternity determination due to the mitochondrial markers used thereby.

Thus, it is the object of the present invention to provide 60 a method for analyzing length polymorphisms in DNA regions which is highly sensitive, achieves reliable results without being time-consuming, is furthermore appropriate for serial examinations and routine testing and can optionally also be carried out automatically.

According to the invention this problem is solved by providing a process for determining identity and kinship of 2

organisms on the basis of length polymorphisms in DNA regions, which process comprises the following steps:

- (a) annealing at least one primer pair to the DNA to be analyzed, wherein one of the molecules of the primer pair is substantially complementary to one of the complementary strands of the DNA flanking a simple or cryptically simple DNA sequence on either the 5' or the 3' side, and wherein the annealing occurs in such an orientation that the synthesis products obtained by a primer-directed polymerization reaction with one of said primers can serve as template for annealing the other primer after denaturation;
- (b) primer-directed polymerase chain reaction; and
- (c) separating and analyzing the polymerase chain reaction products.

In this process the individual primer molecules of the primer pairs are annealed to the DNA region to be analyzed at a distance of 50 to 500 nucleotides apart so that they encompass it at the given distance. Thereby the DNA region to be analyzed is surrounded by the hybridization molecules of the primer pair.

The primer-directed chain reaction is known as such from EP-A2 0 200 362 (1), from EP-A1 0 237 362 (1a) and from (2). It refers to a process for amplification of specific DNA fragments in which a PCR (polymerase chain reaction) is carried out. In this process the specific amplification is achieved by using oligonucleotide primers flanking the target-molecule in an anti-parallel manner. Thereby in a template-dependent extension of the primers by a polymerase DNA fragments are synthesized which themselves are again available as templates for a new cycle of primer extension. The DNA synthesis is performed by heat denaturation of the starting molecules, followed by hybridization of the corresponding primers and by chain extension with a polymerase. By means of a further heat denaturation a following cycle is then performed. Thereby the specifically amplified region grows in an exponential way and finally a fragment detectable by normal gel electrophoresis is formed. The length of this fragment is determined by the length of the primers and the intermediate region and is similar or equal to the sum of the lengths of the primers and the intermediate region. The use of thermostable synthesis components allows control of the process by simple and easily automated heating and cooling cycles.

By "antiparallel flanking" of the target molecule by oligonucleotide primers one understands the hybridizing of one of both primers of a primer pair each to the complementary strands of the target molecule so that the 3' ends of the primer pair point at each other.

In (15) Marx describes different applications of the PCR process.

Rollo et al. describe in (16) the use of the PCR process for distinguishing between various species of the plant pathogenic fungus Phoma.

The use of simple and cryptically simple DNA sequences in the fragment of PCR processes for determining identity and kinship of organisms is not described in any of these references.

SUMMARY OF THE INVENTION

Simple and cryptically simple DNA sequences are repetitive components of all eukaryotic genomes which to some extent can be found also in prokaryotic genomes (6-9). Thereby simple DNA sequences comprise short DNA motifs containing at least one nucleotide and not more than approximately 6 to 10 nucleotides arranged as a dozen to

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approximately one hundred tandem repeats. These simple DNA sequences have been found by hybridization with synthetic DNA sequences and by direct sequencing in all hitherto analyzed eukaryotic genomes and also in the human genome (8, 10). All possible permutations of short motifs 5 can presumably be found therein in different frequency (9). Cryptically simple DNA sequences are characterized by a more than accidentally frequent, but irregularly direct repeat of short DNA motifs (9). Cryptically simple DNA sequences are normally only found indirectly in already sequenced 10 DNA regions by means of a corresponding computer programme. They are, however, at least just as frequent or even more frequent than simple DNA sequences. The simple and cryptically simple DNA sequences are likely to have formed by genomic mechanisms having the tendency to duplicate 15 once more already existing short duplications of any DNA sequence motifs or to partly delete in any DNA sequence motifs longer regions of already existing simple or cryptically simple DNA sequences (8–10). Therefore one can start from the assumption that these regions are usually length 20 polymorphic. The process according to the invention is based on this length polymorphism.

Simple or cryptically simple DNA sequences that are suitable for the process according to the invention can be found with or without a computer programme in DNA 25 sequences that are already known (9). A simple or cryptically simple DNA sequence is suitable for use in the method of the present invention if it has a length of approximately 20 to 300 nucleotides and if it is flanked by random sequences, i.e. DNA sequences without internal repeats. From the region of DNA sequences without internal sequence repeats fragments that flank the simple or cryptically simple DNA sequence are selected. Suitable complementary synthetic oligonucleotides are then prepared which can hybridize to the flanking DNA sequences. An oligonucleotide is suitable for this purpose if its nucleotide composition and its nucleotide sequence can be found most probably only once in the genome to be examined, thus being specific to the DNA region to be individually analyzed.

In the process according to the invention, preferably length polymorphisms of simple or cryptically simple DNA sequences are examined.

When examining length polymorphisms of simple or cryptically simple DNA sequences substantially composed of tri-nucleotide motifs, so-called "slippage"-artifacts are avoided. Slippage-artifacts are more frequently found, for example, in simple or cryptically simple DNA sequences composed of dinucleotide motifs. Thereby reaction products are formed which are shorter than the desired main product (cf. Example 4). These artificial bands are possibly difficult to distinguish from "real" bands which complicates the interpretation of the results. When using simple or cryptically simple tri-nucleotide sequences, these artifacts do not or only rarely occur (cf. Example 3).

In a particularly preferred embodiment of the process according to the invention the simple or cryptically simple DNA sequence is substantially composed of the trinucleotide motif ⁵'CAG^{3'}/⁵'CTG^{3'}.

In the process according to the invention two primer pairs are preferably employed. In a particularly preferred embodiment 2 to 50 primer pairs are employed.

Preferably the primers used in the process according to the invention have a length of 15 to 25 nucleotides.

In a preferred embodiment of the process according to the invention when using several primer pairs the individual

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primer pairs are selected in such a way that the corresponding specific polymerase chain reaction products of the individual primer pairs are separable into individual bands on a suitable gel.

In another preferred embodiment of the process according to the invention the detection of the specific polymerase chain reaction products is carried out by radioactive labelling or by non-radioactive labelling, e.g. with fluorescent dye-stuff.

The labelling of the oligonucleotide pairs can be carried out radioactively or with a fluorescent dyestuff, as described in (12)

Furthermore, kits with which the process according to the invention can be carried out are a subject matter of the present invention. The primers contained therein are optionally labelled radioactively, e.g. with ³⁵S or ¹⁴C, or fluorescently.

The synthesis products obtained in the process according to the invention can be separated using high-resolution gel systems, such as usual sequencing gels. At the same time also the length of the synthesis products can be determined. Polymorphisms which are formed by insertions or deletions of individual or several motifs of the simple or cryptically simple DNA sequence are recognizable by an altered position of the synthesis products in the gel. With an appropriate selection of the primer pairs and with an appropriate resolution capacity of the gel system approximately 20 to 50 independent polymorphic regions can be simultaneously examined. Thus, the identity of an individual can be reliably ascertained due to the individual combination of length distributions of the synthesis products obtained.

If no appropriate simple or cryptically simple DNA sequences are known in the DNA regions to be examined, 35 they can be identified as follows:

A genomic DNA to be examined is subjected to a partial restriction cleavage. Restriction enzymes are used that do normally not cleave in simple or cryptically simple DNA sequences. The DNA fragments obtained are cloned in a suitable vector, e.g. in lambda phage derivatives or in M13-phages and are then screened by usual methods for simple or cryptically simple DNA sequences; cf. (11). The probe molecules used are synthetic DNA molecules containing various permutations of simple or cryptically simple DNA sequences. Thus, hybridizing plaques can be identified. Then the recombinant DNA contained therein can be isolated and characterized by sequencing. The DNA sequence thus obtained can then be screened for DNA sequences which are suitable for the testing procedure according to the invention.

The process according to the invention was carried out with Drosophila-DNA as a model system. As simple and cryptically simple DNA sequences are present in all eukaryotic genomes and to some extent also in prokaryotic genomes, one can assume that the results achieved with the Drosophila model system can also be achieved in the analysis of other genomes, particularly in the examination of the human genome.

Therefore the process according to the invention is suitable for the determination of identity and kinship of organisms, for example of human beings.

In human beings paternity and forensic tests for establishing the identity of delinquents can be carried out with the process according to the invention; cf. also Example 4.

In addition to the determination of identity of individuals the process is also suitable to determine the course of

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hereditary propagation of genetic diseases for which the locus is known and sequenced. For this purpose one or several simple or cryptically simple sequences are selected which are located in or next to the locus to be analyzed. The specific length pattern of these regions is correlated with the 5 mutated locus, as is common practice with known RFLPmarkers; cf. (14). With the families concerned on the basis of this information genetic advice can be given or prenatal diagnosis can be made in a manner analogous to that known for RFLP-markers. The use of the process according to the 10 invention for this purpose makes sense especially because it is based on DNA regions which are polymorphic in all foreseeable probability whereas the RFLP-analysis is dependent on accidentally found variations which are often far away from the locus itself which reduces the certainty of 15 diagnosis.

The process according to the invention is further suitable for determining polymorphisms in simple or cryptically simple DNA sequences of animals and plants. Therefore, in animal breeding, e.g. of horses, dogs or cattle, and kinship 20 to high-grade breeding individuals can be reliably proved.

To sum up, it can be said that the advantage of the process according to the invention vis-a-vis the hitherto known processes lies in its broad applicability, rapid practicability and in its high sensitivity. The amplification step taken for 25 the length polymorphic simple or cryptically simple DNA sequences in the process according to the invention makes it superfluous to take an independent ascertaining step, such as a subsequent hybridization reaction. Therefore the process according to the invention is particularly well suited for 30 automation and for routine testing and serial examination.

BRIEF DESCRIPTION OF THE DRAWINGS

The Figures show:

FIG. 1: Hybridization of a Gene Library with a simple DNA sequence as probe molecule.

Approximately 20,000 independent phage clones were plated out on a 12 by 12 cm plate and hybridized with a probe molecule containing the simple trinucleotide sequence 40 CAG/CTG. 300 to 400 positive signals are thus obtained. The positive signals are recognizable as blackening.

FIG. 2: Sequence (SEQ. ID NO. 5) of the region tested for polymorphism in Example 2.

The regions to which complementary oligonucleotides were synthesized are underlined with a wavy line. The region of the simple DNA sequence is underlined with a double line. The direct repeat of 8 nucleotides is marked with two arrows. The HaeIII-cleavage site is marked in italies

FIG. 3: Analysis of the length variations of 11 wild type strains in Drosophila

The DNA sequences amplified by means of PCR and cleaved with HaeIII are shown in lanes 1 to 11. On the right side a sequencing reaction is shown serving as length marker. The position of the fragments to be expected is marked with arrows on the left side. The positions of the fragment classes additionally observed is marked with lines.

FIG. 4: Test for Reproducibility

Ten independent PCR-reaction mixtures using the DNA preparation "A" of the Drosophila strain No. 3 were applied on the left side, ten independent PCR-reaction mixtures using the DNA preparation "B" of the Drosophila strain No. 3 were applied on the right side. On the very right side 65 marker fragments from a sequencing reaction are applied. All test bands observed are identical.

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FIG. 5: Sequence (SEQ IN NO. 6) of the DNA region used in Example 4.

The regions to which complementary oligonucleotides were synthesized are underlined with a wavy line.

FIG. 6: Paternity analysis in human beings.

The DNA fragments amplified by means of PCR and separated on the gel are shown. In the first lane the DNA of the mother, in the following lanes the DNA of the father to be tested as well as of the three tested children have been applied. In the sixth lane (marked with "C") a control-DNA has been applied which is to indicate only the size categories. The main bands and their size categories are marked on the right side.

DETAILED DESCRIPTION OF THE INVENTION

The examples illustrate the invention.

EXAMPLE 1

Isolation of Clones Containing Simple DNA Sequences

Drosophila-DNA is completely cleaved with the restriction endonuclease EcoRI and the resulting fragments are cloned into the lambda vector 641. A more detailed description of the methods used can be found in (11). By this way a gene library is obtained of which about 20,000 phages are plated out. The corresponding independent plaques are transferred to a nitrocellulose filter and hybridized with a probe molecule containing the simple DNA sequence motif CAG/CTG.

The filters are hybridized and washed at 65° C. The hybridizing solution contains $5 \times \text{SSPE}$, $5 \times \text{Denhardt's}$ solution, 0.1% sodium dodecyl sulfate (SDS) and approximately 1×10^6 cpm/ml of radioactively labelled (^{32}P) DNA as probe molecule. The wash solution contains $2 \times \text{SSPE}$ and 0.1% SDS (the reaction product of Denhardt's solution and SSPE is described in (11)).

About 300 to 400 of the plaques formed show a positive signal; cf. FIG. 1. Some of these plaques are purified, DNA is isolated and sequenced. In the obtained DNA sequences regions can be identified containing the simple DNA sequence CAG/CTG; cf. (7).

EXAMPLE 2

Detection of Length Polymorphisms

For this experiment the DNA sequence illustrated in FIG. 2 and published in (13) was chosen. Two oligonucleotides with the following sequences were synthesized:

Oligonucleotide 1 (SEQ. ID No. 1): 5'-TAAGCTT GGGAATCA-3'

Oligonucleotide 2 (SEQ. ID No. 2): 5'-ATTGAAC TTTGTATC-3'

These DNA sequences are located immediately at the beginning or at the end of the sequence shown in FIG. 2. For use as primers the synthesized oligonucleotides are labelled with ³²P at their 5' end. Then a PCR reaction with the labelled primers is carried out. On the whole 20 cycles are carried out by denaturating at 95° C. for 90 seconds, hybridizing at 45° C. for 90 seconds and then synthesizing at 72° C. for 120 seconds. As DNAs to be examined the genomic DNAs of 11 wild type strains of Drosophila melanogaster from various regions all over the world are employed. These Drosophila wild type strains originally are

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descendants of individual fertilized females and have been collected during the last 10 years. After the PCR reaction the amplified fragments are cleaved with the restriction endonuclease HaeIII. This should normally yield two fragments having a length of 202 and 177 nucleotides, respectively. 5 This step is normally not necessary for routine experiments. Here it only serves to refine the analysis. The resulting fragments are separated on a 5% sequencing gel, the gel is subsequently dried and an X-ray film is exposed to the dried gel. Both DNA fragments expected show a marked polymorphism in the various Drosophila wild type strains. The 202 nucleotide fragment which contains the simple DNA sequence shows four different size categories; see FIG. 3. These size categories are shifted by three nucleotides each. Starting from frameshifts within the repeat of the trinucle- 15 otides this is to be expected. In three cases two different bands appear at the same time; cf. FIG. 3, lanes 5, 8 and 9. This can be explained by the fact that in diploid organisms each locus is to be found twice and can be represented by different alleles (so-called balanced polymorphism). The 20 band of the 177 nucleotide fragment shows three different size categories being 5 or 8 nucleotides apart; cf. FIG. 3. The band which is shorter by 8 nucleotides presumably resulted from a deletion of the repeat of 8 nucleotides labelled in the DNA sequence. The origin of the longer band is unclear. 25 These deletions or insertions correspond to those that can be expected in the region of a cryptically simple DNA

The majority of the strains examined in this simple experiment are readily distinguishable from one another. 30 Only strains 2, 7 and 11 as well as 3 and 4 cannot be distinguished from each other. To distinguish these strains one would therefore employ further primer pairs. For example, 20 to 50 independent DNA regions could be tested, in order to allow a definite identification. As the size 35 categories of the fragments of the individual Drosophila wild type strains are homogenous per se, one has to start from the assumption that the polymorphisms observed are not so frequent that it would no longer be possible to ascertain a kinship. The Drosophila wild type strains all 40 descend from one single original pair and the DNA of several 100 individuals was combined for the test. If a change of the pattern had taken place within these "families", one should expect more than maximal two bands. This is, however, not the case here. From this follows 45 that the length categories observed are stable for at least some dozens of generations.

EXAMPLE 3

Test for Reproducibility

The variations in length observed could also be caused by polymerase errors during the experiment. In order to exclude this possibility and to simultaneously prove the general reproducibility, the experiment carried out in Example 2 is repeated with two different DNA preparations of the Drosophila strains No. 3 in 10 independent reaction mixtures. It can be taken from FIG. 4 that all reaction mixtures lead to the same bands. Similar experiments were also carried out for different loci. In no case, however, a change of the band length could be observed. This shows that the process is reliably reproducible.

EXAMPLE 4

Paternity Test in Human Beings

A primer pair is used which flanks a sequential region from the autosomal human heart muscle actin gene. This 8

sequence contains a simple sequence with a GT/CA dinucleotide repeat structure (FIG. 5). As primers the following oligonucleotides are used:

Primer 1 (SEQ ID No. 3): 5'-CTCCCCC ACACAAGAAG-3'

Primer 2 (SEQ ID No. 4): 5'-TTCCATA CCTGGGAACGA-3'

Primer 2 is labelled at its 5' end with 32P and both oligonucleotides are then used for a PCR reaction. On the whole 25 cycles with a denaturation phase of 1 min. at 94° C., an hybridizing phase of 2 min. at 45° C. and a synthesis phase of 1 min at 72° C. (last synthesis phase for 5 min) are carried out. The reaction products are then separated on a 6% denaturating acrylamide gel, the gel is dried and exposed. The result can be seen in FIG. 6. Each of the tested individuals shows two main bands (for explanation of the further bands, see below), i.e. it is heterozygous for different length variants of this locus. Mother and father have the length variant "109 nt" in common, they do, however, differ in the other variant, with the mother having a "127 nt" and the father a "121 nt" variant. The children must have inherited one of each of these variants from father and mother. For two of the children this is actually the case, whereas the third child (labelled with "?") shows a new "113 nt" variant, which can neither be derived from the mother nor from the tested father. Therefore, one has to assume that this child had another father.

In lane "C" a cloned control-DNA having only one length variant has also been treated. Like the other samples it shows a main band and several secondary bands. The secondary bands are caused by PCR artifacts formed during the amplification. In this context, there are two types of artifacts. The first type results from the fact that the Taq-polymerase has the tendency to attach an additional nucleotide to the completely synthesized DNA strand. Thereby the band is formed which runs a nucleotide above the main band. This effect varies from reaction to reaction, but does not disturb the analysis of the band pattern. A second type of artifact is formed by "slippage" during the amplification process. This leads to the bands which can be seen at the dinucleotide distance below the main bands. These artifact bands could have a disturbing effect on the analysis, if they overlap actual length variants.

Simple sequences with trinucleotide repeat motifs do not show these artifact bands (cf. Example 2), as with these sequences "slippage" occurs less frequently during amplification.

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SEQUENCE LISTING

- (1) GENERAL INFORMATION:
 - (iii) NUMBER OF SEQUENCES: 6
- (2) INFORMATION FOR SEQ ID NO: 1:
 - - (i) SEQUENCE CHARACTERISTICS:(A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TAAGCTTGGG AATCA 15

- (2) INFORMATION FOR SEQ ID NO: 2:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single (D) TOPOLOGY: linear
 - (-, -----
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATTGAACTTT GTATC

- (2) INFORMATION FOR SEQ ID NO: 3:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CTCCCCCACA CAAAGAAG 18

- (2) INFORMATION FOR SEQ ID NO: 4:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTCCATACCT GGGAACGA 18

11 12

-c				

(i) SEQUENCE CHARACTERISTICS: (A) LEMOTH: 379 base pairs (B) TYPE: nucleic acid (C) STRANDEDENSS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5: TRAGGTRGG AATCANCTCG CCGACGGGCA GCGATATGGG CATCATGCTC GCCCCGCCC (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5: TRAGGTRGGA AATCANCTGG CAGACGGGCA GCGATATGGC CAGCAACAG CAGCAGCAGC AANTCCTGGAA GAATCATGCAAA CGATATCACC CCAGCAACAG CAGCAGCAGC AACGCAGCA ACAGCAGCAA CATCAGCAGC AGCAACAGC AGCAGACAG CAGCAGCAGC AACAGCAGCA ACAGCAGCAA CATCAGCAGC AGCAACAGC GCAGCAACAG CAGCAGCAGC AACAGCAGCA CAGCAGCAG AGCATCAGGAG AGCATCAGGA GGGCTAGACAG CTGAATGGAT TTTGTGGATC TCCGGGTAAG TGGTCACTCA TGATGGACT TATGGACTC CTAACTAGCT 300 AACACATATCAT TCTACCATCC CAACTTGCAG ACTCATTTCA CTCGGGTCAA ATGAATCCCC (I) SEQUENCE CHARACTERISTICS: (A) SEQUENCE CHARACTERISTICS: (B) TYPE: nucleic acid (C) STRANDEDENSS: single (D) TOPOLOGY: Linear (ii) MOLECULE TYPE: DNA (genomic) (ix) FEATURE: (A) NAME/KEY: repeat_region (B) LOCATTON: 5887 (D) OTHER INFORMATION: /rpt.type= "other"	(2) INFORMATION FOR SEQ ID NO: 5:				
(xi) SEQUENCE DESCRIPTION: SEQ ID No: 5: TARGCTTGGG ARTCATCTG CCGACGGGCA GCGATATGGG CATCATGCTC GCCCCGCCCC	(A) LENGTH: 379 base pairs(B) TYPE: nucleic acid(C) STRANDEDNESS: single				
TRANSCTTEGG ANTCATCTCG CCGACGGGCA GCGATATGGG CATCATGCTC GCCCCGCCCC	(ii) MOLECULE TYPE: DNA (genomic)				
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ARCAGCAGCA ACAGCAGCAA CATCAGCAGC AGCAACAGCA GCAGCAACAG CAGCAGCAGC AACAGCAGCA GCAACTCGGA GGCCTGGAGT TCGGTTCAGA GGGCTTGGAC CTGAATGGAT TTTGTGGATC TCCGGGTAAG TGGTCACTCA TGATGGACTC TATGGACTCG CTAACTAGCT AACTAATCAT TCTACCATCC CAACTTGCAG ACTCATTTCA CTCGGGTCAA ATGAATCCGC CCTCGATACA AAGTTCAAT (2) INFORMATION FOR SEQ ID NO: 6: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 107 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (ix) FEATURE: (A) NAME/KEY: repeat_region (B) LOCATION: 5887 (D) OTHER INFORMATION: /rpt_type= "other"	TAAGCTTGGG AATCATCTCG CCGACGGGCA GCGATATGGG CATCATGCTC GCCCCGCCCC	60			
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AACTAATCAT TCTACCATCC CAACTTGCAG ACTCATTCA CTCGGGTCAA ATGAATCCGC AACTAATCAT TCTACCATCC CAACTTGCAG ACTCATTTCA CTCGGGTCAA ATGAATCCGC 360 CCTCGGATACA AAGTTCAAT (2) INFORMATION FOR SEQ ID NO: 6: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 107 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (ix) FEATURE: (A) NAME/KEY: repeat_region (B) LOCATION: 5887 (D) OTHER INFORMATION: /rpt_type= "other"	AGCAGCAGCA ACAGCAGCAA CATCAGCAGC AGCAACAGCA GCAGCAACAG CAGCAGCAGC	180			
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(2) INFORMATION FOR SEQ ID NO: 6: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 107 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (ix) FEATURE: (A) NAME/KEY: repeat_region (B) LOCATION: 5887 (D) OTHER INFORMATION: /rpt_type= "other"	TTTGTGGATC TCCGGGTAAG TGGTCACTCA TGATGGACTC TATGGACTCG CTAACTAGCT	300			
(2) INFORMATION FOR SEQ ID NO: 6: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 107 base pairs (B) TYPE: nucleic acid (C) STRANDEDMESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (ix) FEATURE: (A) NAME/KEY: repeat_region (B) LOCATION: 5887 (D) OTHER INFORMATION: /rpt_type= "other"	AACTAATCAT TCTACCATCC CAACTTGCAG ACTCATTTCA CTCGGGTCAA ATGAATCCGC	360			
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 107 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (ix) FEATURE: (A) NAME/KEY: repeat_region (B) LOCATION: 5887 (D) OTHER INFORMATION: /rpt_type= "other"	CCTCGATACA AAGTTCAAT	379			
(B) LOCATION: 5887 (D) OTHER INFORMATION: /rpt_type= "other"	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 107 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (ix) FEATURE:				
(A) NAME/KEY: - (B) LOCATION: 5887 (D) OTHER INFORNATION: /label= TG_DINUCLEOTIDE /note= "THIS STRETCH OF TG DINUCLEOTIDES RANGES IN SIZE FROM 15 DINUCLEOTIDES TO 25 DINUCLEOTIDES ((TG)15 TO 25)" (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6: CTCCCCCCACA CAAAGAAGTT CTGTTCTCTT CCCTCTACCT TGATGAATGC ACTGTGATGT 60	(B) LOCATION: 5887(D) OTHER INFORMATION: /rpt_type= "other"				
CTCCCCCACA CAAAGAAGTT CTGTTCTCTT CCCTCTACCT TGATGAATGC ACTGTGATGT 60	(A) NAME/KEY: - (B) LOCATION: 5887 (D) OTHER INFORMATION: /label= TG_DINUCLEOTIDE /note= "THIS STRETCH OF TG DINUCLEOTIDES RANGES IN SIZE FROM 15 DINUCLEOTIDES TO 25 DINUCLEOTIDES				
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:				
GTGTGTGTGT GTGTGTGACT CGTTCCCAGG TATGGAA 107	CTCCCCCACA CAAAGAAGTT CTGTTCTCTT CCCTCTACCT TGATGAATGC ACTGTGATGT	60			
	GTGTGTGTGT GTGTGTGT GTGTGTGACT CGTTCCCAGG TATGGAA	107			

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We claim:

1. A method for determining length polymorphisms in a simple or cryptically simple sequence in one or more DNA regions of one or more subjects, which comprises:

- a) providing at least one DNA sample, comprising a 55 template DNA having a nucleotide sequence that includes a simple or cryptically simple sequence comprising trinucleotide repeats, from at least one subject;
- b) annealing at least one primer pair to the template DNA of each of said DNA samples, wherein said primer pair 60 is composed of a first primer complementary to a nucleotide sequence flanking the simple or cryptically simple DNA sequence on the 5' side of said simple or cryptically simple DNA sequence and a second primer complementary to a nucleotide sequence flanking the 65 simple or cryptically simple DNA sequence on the 3' side of said simple or cryptically simple DNA
- sequence; wherein said first and second primers each anneal to a single site in said template DNA and the sequence of the template DNA between the sites where said primers anneal is 50 to 500 nucleotides in length;
- c) performing at least one primer-directed polymerase chain reaction upon said template DNA having said primers annealed thereto, so as to form at least one polymerase chain reaction product;
- d) separating the products of each polymerase chain reaction according to their lengths; and
- e) analyzing the *lengths of the* separated products to determine the length polymorphisms of the simple or cryptically simple sequences.
- [2. The method according to claim 1, wherein each simple or cryptically simple DNA sequence comprises at least one trinucleotide motif.]

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- 3. The method according to claim 1, wherein [each simple or cryptically simple DNA sequence contains] the *trinucle-otide repeat* motif is CAG/CTG.
- **4**. The method according to claim **1**, wherein at least 2 primer pairs are used.
- 5. The method according to claim 1, wherein between 2 and 50 primer pairs are used.
- 6. The method according to claim 1, wherein each of the primers has a length ranging between 15 and 25 nucleotides.
- 7. The method according to claim [2] 1, wherein the 10 annealing position of the primers of each pair is selected such that each of the primer-directed polymerase chain reaction products are separable one from the other as individual bands on a suitable electrophoretic gel.
- 8. The method according to claim 1, wherein the product 15 of each primer-directed polymerase chain reaction is labelled by a radioactive label.
- **9.** The method according to claim **1**, wherein the product of each primer-directed polymerase chain reaction is labelled by a non-radioactive label.
- 10. The method according to claim 9, wherein said non-radioactive label is a fluorescent label.
- 11. The method according to claim 1, wherein said simple or cryptically simple DNA sequence is located adjacent to or within a genetically defined locus such that said simple or 25 cryptically simple DNA sequence can serve as a marker for said locus.
- 12. A kit for performing the method of claim 1, comprising:
 - a) at least one vessel containing an equimolar mixture of ³⁰ primers constituting between 1 and 50 of said primer pairs;
 - b) a vessel containing a polymerizing enzyme suitable for performing a primer-directed polymerase chain reaction;
 - c) a vessel containing the deoxynucleotide triphosphates adenosine, guanine, cytosine and thymidine;
 - d) a vessel containing a buffer solution suitable for performing a polymerase chain reaction, or a concentrate of said buffer solution;
 - e) a vessel containing a template DNA that has a nucleotide sequence including a simple or cryptically simple sequence for assaying positive performance of the method, wherein each simple or cryptically simple 45 DNA sequence comprises at least one trinucleotide motif.
- 13. The kit of claim 12, wherein at least one primer of each primer pair is labelled with a fluorescent or a radioactive label.
- 14. The method of claim 1, wherein the kinship of at least two subjects is determined by comparing length polymorphisms determined in step (e).
- 15. A method for analyzing length polymorphisms in at least one locus in an DNA sample obtained from at least one 55 subject, wherein said DNA sample comprises a DNA template having at least one locus comprising a simple or cryptically simple DNA sequence, said method comprising:
 - a) annealing said DNA template with at least one pair of primers, wherein said primer pair is composed of a first 60 primer complementary to a nucleotide sequence flanking said simple or cryptically simple DNA sequence on the 5' side of said simple or cryptically simple DNA sequence and a second primer complementary to a nucleotide sequence flanking the simple or cryptically 65 simple DNA sequence on the 3' side of said simple or cryptically simple DNA sequence; wherein said first

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- and second primers each anneal to a single site in said DNA template and wherein the annealing sites are separated by 50 to 500 nucleotides of template DNA;
- b) performing at least one primer-directed polymerase chain reaction upon said template DNA having said primers annealed thereto, so as to form at least one polymerase chain reaction product;
- c) separating the products of each polymerase chain reaction according to their lengths; and
- d) analyzing the lengths of the separated products to determine the length polymorphisms of said simple or cryptically simple sequences;
- wherein said simple or cryptically simple sequence has a repeat length of 3 to 10 nucleotides.
- 16. The method of claim 15, wherein said simple or cryptically simple sequence has a repeat length of 3 to 6 nucleotides.
- 17. The method of claim 15, wherein said simple or cryptically simple sequence has a repeat length of 3 nucleotides.
- 18. The method of claim 15, wherein at least two primer pairs are used.
- 19. The method of claim 15, wherein the kinship of at least two subjects is determined by comparing the length polymorphisms determined in step (d).
- 20. The method of claim 16, wherein the kinship of at least two subjects is determined by comparing the length polymorphisms determined in step (d).
- 21. The method of claim 15, further comprising (e) comparing the length polymorphisms determined in step (d) to numerical values of length polymorphisms for said at least one locus.
- 22. The method of claim 16, further comprising (e) comparing the length polymorphisms determined in step (d) to numerical values of length polymorphisms for said at least one locus.
- 23. The method of claim 15, wherein 2 to 50 primer pairs are used.
- 24. The method according to claim 15, wherein each of the primers has a length of 15 to 25 nucleotides.
- 25. The method according to claim 15, wherein the annealing position of the primers of each pair is selected such that each of the products of the primer-directed polymerase chain reaction is separable one from the other as individual bands on a suitable electrophoretic gel.
- 26. The method according to claim 15, wherein the product of each primer-directed polymerase chain reaction is labeled by a radioactive label.
- 27. The method according to claim 15, wherein the product of each primer-directed polymerase chain reaction 50 is labeled by a non-radioactive label.
 - 28. The method according to claim 27, wherein said non-radioactive label is a fluorescent label.
 - 29. The method according to claim 15, wherein said simple or cryptically simple DNA sequence is located adjacent to or within a genetically defined locus such that said simple or cryptically simple DNA sequence can serve as a marker for said locus.
 - 30. The method of claim 15, wherein said simple or cryptically simple sequence has a repeat length of 3 to 6 nucleotides, each of said primers has a length of 15 to 25 nucleotides and the kinship of at least two subjects is determined by comparing the length polymorphisms determined in step (d).
 - 31. The method of claim 15, wherein said simple or cryptically simple sequence has a repeat length of 3 to 6 nucleotides, each of said primers has a length of 15 to 25 nucleotides and wherein 2 to 50 primer pairs are used.

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- 32. The method of claim 29, wherein said simple or cryptically simple sequence has a repeat length of 3 to 6 nucleotides, each of said primers has a length of 15 to 25 nucleotides and 2 to 50 primer pairs are used.
- 33. The method of claim 30, wherein said simple or 5 cryptically simple sequence has a repeat length of 3 nucleotides.
- 34. The method of claim 32, wherein said simple or cryptically simple sequence has a repeat length of 3 nucleotides
- 35. A kit for performing analysis of polymorphism in simple or cryptically simple sequences, comprising:
 - a) at least one vessel containing a mixture of primers constituting 1 to 50 primer parts; wherein each of said primer pairs is composed of a first primer complementary to a nucleotide sequence flanking said simple or cryptically simple DNA sequence on the 5' side of said simple or cryptically simple DNA sequence and a second primer complementary to a nucleotide sequence flanking the simple or cryptically simple DNA sequence on the 3' side of said simple or cryptically simple DNA sequence; wherein said first and second primers each anneal to a single site in said DNA template and wherein the annealing sites are separated by 50 to 500 nucleotides of template DNA;
 - b) a vessel containing a template DNA that has a nucleotide sequence including a simple or cryptically simple sequence for assaying positive performance of the method.
- 36. The kit of claim 35, wherein at least one primer of ³⁰ each primer pair is labeled with a fluorescent or a radioactive label.
- 37. The kit of claim 35, wherein said simple or cryptically simple sequence has a repeat length of 3 to 6 nucleotides and each of said primers has a length of 15 to 25 nucleotides.
- 38. The kit of claim 35, wherein said simple or cryptically simple DNA sequence is located adjacent to or within a genetically defined locus such that said simple or cryptically simple DNA sequence can serve as a marker for said locus.
- 39. The kit of claim 38, wherein at least one primer of 40 each primer pair is labeled with a fluorescent or a radioactive label.
- 40. A method for determining length polymorphisms in a simple or cryptically simple sequence in one or more DNA regions of one or more subjects, which comprises:
 - a) providing at least one DNA sample, comprising a template DNA consisting essentially of a nucleotide sequence that includes i) a simple or cryptically simple sequence having a trinucleotide repeat motif and ii) nucleotide sequences flanking the simple or cryptically simple sequence, from at least one subject;
 - b) annealing at least one primer pair to the template DNA of each of said DNA samples, wherein said primer pair is composed of a first primer complementary to the nucleotide sequence flanking the simple or cryptically simple DNA sequence on the 5' side of said simple or cryptically simple DNA sequence and a second primer complementary to the nucleotide sequence flanking the simple or cryptically simple DNA sequence on the 3' side of said simple or cryptically simple DNA sequence; wherein said first and second primers each anneal to a single site in said template DNA and the

sequence of the template DNA between the sites where said primers anneal is 50 to 500 nucleotides in length;

- c) performing at least one primer-directed polymerase chain reaction upon said template DNA having said primers annealed thereto, so as to form at least one polymerase chain reaction product;
- d) separating the products of each polymerase chain reaction according to their lengths;
- e) analyzing the lengths of the separated products to determine the length polymorphisms of the simple or cryptically simple sequences.
- 41. A method for analyzing polymorphism in at least one locus in an DNA sample comprising a DNA template, said method comprising:
 - a) annealing said DNA template with at least one pair of primers, wherein said primer pair is composed of a first primer complementary to a nucleotide sequence flanking said simple or cryptically simple DNA sequence on the 5' side of said simple or cryptically simple DNA sequence and a second primer complementary to a nucleotide sequence flanking the simple or cryptically simple DNA sequence on the 3' side of said simple or cryptically simple DNA sequence; wherein said first and second primers each anneal to a single site in said DNA template and wherein the annealing sites are separated by 50 to 500 nucleotides of template DNA;
 - b) performing at least one primer-directed polymerase chain reaction upon said template DNA having said primers annealed thereto, so as to form at least one polymerase chain reaction product;
 - c) separating the products of each polymerase chain reaction product according to their lengths; and
 - d) analyzing the lengths of the separated products to determine the length polymorphisms of said simple or cryptically simple sequences,
 - wherein said DNA template includes at least one sequence consisting essentially of a simple or cryptically simple DNA sequence having a repeat motif length of 3 to 10 nucleotides and nucleotide sequences flanking said simple or cryptically simple DNA sequence effective for annealing said at least one pair of primers.
- 42. A kit for analyzing polymorphism in at least one locus in an DNA sample, comprising:
 - a) at least one vessel containing a mixture of primers constituting between 1 and 50 of said primer pairs;
 - b) a vessel containing a polymerizing enzyme suitable for performing a primer-directed polymerase chain reaction;
 - c) a vessel containing the deoxynucleotide triphosphates adenosine, guanine, cytosine and thymidine;
 - d) a vessel containing a buffer solution for performing a polymerase chain reaction;
 - e) a vessel containing a template DNA comprising i) a simple or cryptically simple nucleotide sequence having a repeat motif length of 3 to 10 nucleotides and ii) nucleotide sequences flanking said simple or cryptically simple nucleotide sequence that are effective for annealing at least one pair of said primers, for assaying positive performance of the method.

* * * * *

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CERTIFICATE OF COMPLIANCE

The undersigned certifies that this brief complies with the type-volume limitations of Fed. R. App. P. 32(a)(7)((B). This brief contains 13,988 words as calculated by the "Word Count" feature of Microsoft Word 2010, the word processing program used to create it.

The undersigned further certifies that this brief complies with the typeface requirements of Fed. R. App. P. 32(a)(5) and the type style requirements of Fed. R. App. P. 32(a)(6). This brief has been prepared in a proportionally spaced typeface using Microsoft Word 2010 in Times New Roman 14 point font.

Dated: July 12, 2013 /s/ Edward R. Reines

Edward R. Reines

Counsel for Defendants-Appellants

CERTIFICATE OF SERVICE

This is to certify that on July 12, 2013, copies of the foregoing were served via electronic mail on the following counsel for Plaintiff-Cross Appellant Promega Corporation:

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